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# THE CARDIOVASCULAR CAROTID SINUS REFLEX

CHARLES F. CODE, W. T. DINGLE AND V. H. K. MOORHOUSE

*From the Department of Physiology, The Mayo Foundation, Rochester, Minnesota and the Department of Physiology, University of Manitoba, Winnipeg, Manitoba, Canada*

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This research was undertaken to study thoroughly the pathway of the cardiovascular sinus reflex in the dog.

All the principal nerves in the region of the carotid sinus have been mentioned in the literature as supplying fibers to that sinus. Hering (8), in his original publications, first mentioned that the sinus reflex was transmitted through nerves which pass from the carotid sinus to the superior cervical ganglion of the sympathetic nervous system. He (9) later described a branch from the glossopharyngeal nerve, which he named the sinus nerve. On the basis of his experiments, Hering believed that the reflex from the carotid sinus was transmitted by the sinus nerve.

De Castro (2), (3) has attacked the problem from the morphologic standpoint. Because the branch from the glossopharyngeal nerve supplies fibers to both the carotid sinus and the carotid body, De Castro preferred to call it the intercarotid nerve. He has found that after section of the glossopharyngeal nerves, including its intercarotid branch, the specialized receptors are still found in the carotid sinus. In his experiments the specialized nerve endings in the carotid sinus remained unaltered, after removal of the superior cervical ganglion. He has emphasized the importance of branches from the vagus nerve and from the ganglion nodosum. He was of the opinion that afferent impulses liberated from the carotid sinus reach the medullary centers partly through the glossopharyngeal nerve but more particularly by way of the vagus nerve.

Daniélopolu and his co-workers (4) have postulated a threefold nerve supply; namely, a branch from the glossopharyngeal nerve, a branch from the vagus, and a branch from the superior cervical ganglion of the sympathetic nervous system. They said that all three nerves take part in

the transmission of the reflexes of the carotid sinus. It also was the opinion of Heymans, Bouckaert, and Regniers that the afferent fibers from the carotid sinus reach the centers not only through the glossopharyngeal nerve but also through branches which pass from the carotid sinus to the vagus and sympathetic nerves. Sampson Wright has said that connections may perhaps be made not only with the superior cervical ganglion but also with the hypoglossal nerve.

Kahn in his observations on the dog found fibers which reached the sinus by way of the glossopharyngeal nerve and also through the pharyngeal branch of the vagus nerve. Kahn said that the main afferent pathway from the carotid sinus is through the glossopharyngeal nerve but that on some occasions the vagus nerve may play a part. Moissejeff has found that distention of the carotid sinus may occasionally lower the blood pressure after section of the sinus nerve. He believed this effect was the result of the presence of afferent fibers in the sympathetic nerves which supply the carotid sinus.

Garard and Billingsly have carefully dissected the intercarotid plexus of five dogs. Fibers entered this plexus from the inferior pharyngeal branch of the glossopharyngeal nerves, from the middle part of the superior cervical ganglion, and in two animals from the vagosympathetic trunk. In one instance, they traced these last fibers centrally and found that they entered the superior cervical ganglion.

ANATOMIC CONSIDERATIONS. *Experimental procedure.* All our observations were made on the dog. The procedure included a detailed dissection of the region of the carotid sinus in twenty-five animals.

The glossopharyngeal nerve leaves the cranium by the jugular foramen. In our dissections, we found that shortly after issuing from the foramen the glossopharyngeal nerve is divided into an anterior and a posterior division by a minute vessel which arises in the region of the bifurcation of the common carotid artery. Both the anterior and posterior divisions divide into ascending and descending branches (fig. 1). The descending branch of the posterior division consists of a long slender nerve which passes to the carotid sinus. This is the sinus nerve of Hering. In its course downward, it usually communicates with the large branch from the superior cervical ganglion (fig. 1, right side). On reaching the neighborhood of the carotid sinus it is distributed mainly to the posterior aspect of the carotid sinus and carotid body. Not infrequently, however, a small twig curls around beneath the internal carotid artery and ends on the anterior aspect of the carotid sinus and carotid body.

From approximately the middle of the lateral aspect of the superior cervical ganglion, there arises a large branch which passes downward and laterally toward the bifurcation of the common carotid artery, and then turns upward and laterally in close association with the external carotid

artery. In its course, therefore, it roughly describes a "u," and in our experiments we called it the "sympathetic u" (fig. 1). A branch of considerable size passes from the sympathetic u to the carotid body. Occasionally, this branch arises close to the superior cervical ganglion (fig. 1, right side), but more frequently it is given off just above the carotid sinus

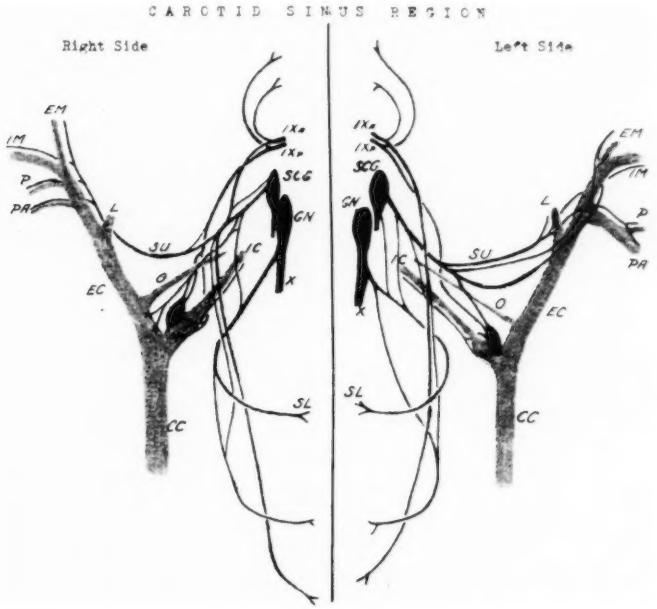


Fig. 1. Diagram of the usual distribution of nerves in the region of the carotid sinus. The drawing of the right or left sinus regions is not necessarily the typical arrangement of the nerves on that side. The two diagrams are shown to indicate some of the most marked variations which occur irrespective of the side. *CC*, common carotid artery; *EC*, external carotid artery; *EM*, external maxillary artery; *GN*, ganglion nodosum; *IC*, internal carotid artery; *IM*, internal maxillary artery; *L*, lingual artery; *O*, occipital artery; *P*, branch to parotid gland; *PA*, posterior auricular artery; *SCG*, superior cervical ganglion; *SL*, superior laryngeal nerve; *SU*, sympathetic "U"; *IXa*, anterior division glossopharyngeal nerve; *IXp*, posterior division glossopharyngeal nerve; *X*, vagus nerve.

(fig. 1, left side). The branch from the sympathetic u, which goes to the carotid body, and the branch from the glossopharyngeal nerve, which gives fibers to both the carotid body and the carotid sinus, were identified in every specimen which we dissected.

Smaller and more difficult to find is a minute, somewhat variable, twig

which passes upward from the region of the carotid sinus, along the medial side of the internal carotid artery, and accompanies this vessel into the skull. It may communicate with the ganglion nodosum of the vagus nerve. This is the only connection between the carotid sinus and the vagus nerve which we were able to find in our dissections. Not infrequently, we were unable to identify either the nerve or the communication with the ganglion nodosum.

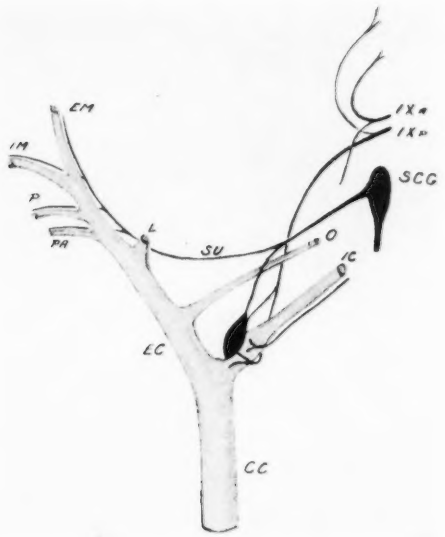


Fig. 2. Simplified diagram of region of carotid sinus. *CC*, common carotid artery; *EC*, external carotid artery; *EM*, external maxillary artery; *IC*, internal carotid artery; *IM*, internal maxillary artery; *L*, lingual artery; *O*, occipital artery; *P*, branch to parotid gland; *PA*, posterior auricular artery; *SCG*, superior cervical ganglion; *SU*, sympathetic "U"; *IXa*, anterior division of glossopharyngeal nerve; *IXp*, posterior division of glossopharyngeal nerve.

In our dissections, three nerves were found actually to end in the region of the carotid sinus (fig. 2). They were the sinus nerve from the glossopharyngeal nerve, the branch from the sympathetic nervous system, and the branch along the medial side of the internal carotid artery. The next step in our research was to acquire some knowledge of the part played by these three nerves in the production of the reflexes of the carotid sinus.

PHYSIOLOGIC CONSIDERATIONS. *Experimental procedure.* The function of the three nerves which we found proceeding from the carotid sinus has been investigated by means of three methods: 1, electric stimulation

of the nerves which pass from the sinus; 2, stimulation by raising and lowering the pressure in the isolated sinus; and 3, survival denervation experiments.

In all acute experiments, anesthesia was secured with morphine-urethane or with the intravenous administration of sodium amytal. Electric stimulation was supplied by a Harvard inductorium. The blood pressure in either the femoral artery or the common carotid artery was recorded with the use of a solution of heparin or sodium citrate as the anticoagulant. A pneumographic record of the respiratory rate was made in the majority of experiments. The mean blood pressure only was calculated. The heart rate was determined for five, ten, or more seconds. Each experiment was

TABLE 1

*Comparative effects of electric stimulation of sinus and sympathetic nerves**

| STRENGTH OF STIMULUS, CM. | NERVE STIMULATED | NUMBER OF EXPERIMENTS | NUMBER OF STIMULATIONS | AVERAGE EFFECT ON BLOOD PRESSURE, MM. OF MERCURY | AVERAGE EFFECT ON HEART RATE, BEATS PER MINUTE |
|---|---|---|---|---|---|
| 11 | Sinus nerve | 1 | 1 | −32 | −48 |
| 11 | Sympathetic nerve | 1 | 1 | 0 | 0 |
| 10 | Sinus nerve | 4 | 4 | −42 | −58 |
| 10 | Sympathetic nerve | 3 | 3 | 0 | −2 |
| 8 | Sinus nerve | 4 | 6 | −62 | −58 |
| 8 | Sympathetic nerve | 4 | 4 | −3 | −19 |
| 6 | Sinus nerve | 6 | 12 | −63 | −113 |
| 6 | Sympathetic nerve | 6 | 15 | −30 | −44 |

* Effect of stimulation is expressed as the actual or average difference between the blood pressure or heart rate immediately before stimulation and during stimulation. The strength of electric stimulation is expressed in centimeters, indicating the position of the secondary coil of the inductorium.

performed on a different animal. The nerve from the carotid sinus, which joins the glossopharyngeal nerve, is referred to in the tables and the following report as the sinus nerve.

*Electric stimulation.* Electric stimulation of the small nerve along the medial side of the internal carotid artery had practically no effect on the pulse rate or blood pressure. Electric stimulation alone eliminated this minute variable twig.

We next studied the comparative effects of varying strengths of electric stimulation on the sinus nerve and the sympathetic nerve (table 1). This group of experiments clearly indicated the greater importance of the sinus nerve. It was excited by weaker stimuli than was the sympathetic nerve,

and also produced a greater effect on the pulse rate and blood pressure than did the sympathetic nerve.

We have already pointed out that communications frequently pass between the sinus nerve and the sympathetic nerves. It seemed probable, therefore, that the depressor responses of the sympathetic nerve were the result, in part at least, of fibers derived from the sinus nerve. The sympathetic nerve was therefore stimulated before and after section of the sinus nerve above the point of its communication with the sympathetic

TABLE 2

*Electric stimulation of the sympathetic nerve before and after section of sinus nerve** (Strength of stimulation 6 cm. in all cases)

| EXPERIMENT | ELECTRIC STIMULATION OF | NUMBER OF STIMULATIONS | AVERAGE EFFECT ON BLOOD PRESSURE, MM. OF MERCURY | AVERAGE EFFECT ON HEART RATE, BEATS PER MINUTE |
|---|---|---|---|---|
| A | Sympathetic nerve before section of sinus nerve | 1 | −38 | −66 |
| | Sympathetic nerve after section of sinus nerve | 2 | −24 | −24 |
| B | Sympathetic nerve before section of sinus nerve | 2 | −100 | −75 |
| | Sympathetic nerve after section of sinus nerve | 1 | +2 | +6 |
| C | Sympathetic nerve before section of sinus nerve | 1 | −28 | −36 |
| | Sympathetic nerve after section of sinus nerve | 1 | +6 | +6 |
| D | Communicating twig between sinus nerve and sympathetic | 1 | −24 | −50 |

* Effect of stimulation is expressed as the actual or average difference between the blood pressure or heart rate immediately before stimulation and during stimulation. The strength of electric stimulation is expressed in centimeters, indicating the position of the secondary coil of the inductorium.

nerve (table 2). It became apparent that the responses of the sympathetic nerve to stimulation were greatly reduced or abolished by section of the sinus nerve. It is suggested, therefore, that any depressor effects produced by stimulation of the sympathetic nerve were the result of depressor fibers carried to the sympathetic nerve by communication with the sinus nerve, or were caused by the spreading of the current along communicating branches to the glossopharyngeal trunk, or to the adjacent ganglion nosodum and the vagus nerve.

*Stimulation by means of raising and lowering the pressure in the iso-*

*lated carotid sinus.* In this group of experiments the carotid sinus was prepared for stimulation according to the technic first devised by Moissejeff. A cannula for the injection of fluid was placed in the common carotid artery just below its bifurcation, and all the vessels above the carotid sinus were then ligated. The fluid injected was either physiologic saline solution or Ringer's solution, at body temperature. A large syringe was used to inject these solutions. In a number of experiments the actual pressure exerted was checked by means of a mercury manometer. We found that the pressure delivered by the use of the syringe invariably exceeded the pressure which Koch found gave the maximal response in the dog. This method of stimulation probably explains the uniformity of the cardiovascular responses in our experiments.

The procedure in this type of experiment was as follows: The pressure within the carotid sinus was raised for fifteen to twenty seconds and then immediately lowered to zero or almost zero. This was repeated two or more times with an ample period for recovery between each trial. Then, one of the nerves to the sinus was cut and the effect of varying the pressure in the sinus again was tested. Finally, the second nerve was cut and the effect of this procedure was tested. Since electric stimulation of the small nerve accompanying the internal carotid artery had proved ineffective, section of this nerve was not included in this group of experiments.

Observations have been made on fourteen sinus regions in twelve different dogs. Our results have been consistent throughout the series. Section of the sympathetic nerve alone had little or no effect on the cardiovascular components of the carotid sinus reflex. When the sinus nerve was cut first and the sympathetic nerve left intact, raising or lowering the pressure within the carotid sinus no longer affected either the blood pressure or the heart rate. In one of ten experiments, section of the sinus nerve did not completely abolish the cardiovascular reflex. In this experiment, the sinus nerve was cut close to the carotid sinus. It is possible that communicating branches were still intact. For this reason, in subsequent experiments in which denervation was required, the sinus nerve was evulsed. In eight consecutive observations, this procedure successfully abolished the cardiovascular sinus reflex.

This group of experiments allows us to conclude that the cardiovascular components of the sinus reflex are conducted solely by the sinus nerve.

Hering (10) originally maintained that the regulatory action of the carotid sinus on the circulation was produced solely by means of a depressor mechanism. Evidence has, however, been submitted, which suggests that pressor reflexes also play a part in the responses of the carotid sinus (4), (5) (11). Tournade and Malméjac found that electric stimulation of the sinus nerve caused a rise of blood pressure in some cases especially if the vagi had been sectioned previously. We have stimulated the nerves

to the carotid sinus with varying strengths of electric current, before and after vagal section. Stimulation of the small nerve which accompanies the internal carotid artery did not once produce a rise of blood pressure associated with an increased pulse rate. With all nerves intact, thirty stimulations of the sympathetic nerve failed to reveal a single typical pressor response. During twenty stimulations of the sympathetic nerve following bilateral vagal section, there was never an increase of more than six beats per minute in the heart rate, or a rise of more than 6 mm. of mercury in the blood pressure.

Our observations have recently been extended. They now include the results of stimulating fourteen carotid sinus nerves before and after bilateral vagal section, in eleven dogs. On no occasion has electric stimulation of the sinus nerve caused a typical pressor reflex. When all data are considered, we find that, out of approximately ninety cases in which the sinus nerve was stimulated, an appreciable rise of blood pressure was recorded in only three cases, while the heart rate in no instance increased more than six beats per minute.

Stimulation of carotid sinus by distention on no occasion produced a significant pressor response. Our experiments, therefore, lend support to the view that the carotid sinus operates entirely through a depressor reflex mechanism.

Survival denervation experiments. In five animals under ether anesthesia, the sinus nerve on one side was evulsed surgically. The nerve on the opposite side was left intact. We have utilized a simple approach to the sinus nerve. It depends on the fact that the sinus nerve lies in an exceedingly dense plexus in the neighborhood of the carotid sinus. No effort was made to isolate the nerve in this region. It was sought for at its point of origin from the glossopharyngeal nerve. Here, it lies separate and may be found without difficulty (fig. 1).

Through a median incision on the ventral aspect of the neck, the lingual vein was divided between ligatures. The hypoglossal nerve and artery were retracted caudally, and the dissection was continued upward into the angle between the mylohyoid and digastric muscles. These were separated and the external carotid artery was drawn laterally. Two muscles, the hypoglossus and the middle constrictor of the pharynx, were brought into view. The lower or caudal muscle is the middle constrictor of the pharynx. It is the important guide to the glossopharyngeal nerve. Curling upward over its lower border is a branch of the glossopharyngeal nerve. This branch was elevated and drawn medially. Using a pointed hemostat for dissection, the dorsolateral aspect of the posterior division of the glossopharyngeal nerve was exposed and followed to its point of exit from the skull. Arising from the caudal border of this division of the glossopharyngeal is the sinus nerve. It is the first branch of the glossopharyn-

geal nerve. It may arise 2 cm. from the point where the glossopharyngeal nerve leaves the cranium, or it may appear as a separate nerve at the jugular foramen. The sinus nerve was dissected for a short distance. A small hemostat was placed at the caudal end of the dissected portion. The nerve was then sectioned below the hemostat. By winding the nerve

TABLE 3

*Effect of raising pressure in carotid sinus following evulsion of sinus nerve**

Right carotid sinus nerve evulsed at operation 33 to 240 days prior to the experiment. Left carotid sinus nerve evulsed at the time of the experiment.

| EXPERIMENT | DAYS AFTER OPERATION | PRESSURE RAISED IN CAROTID SINUS | NUMBER OF TRIALS | AVERAGE EFFECT ON BLOOD PRESSURE, MM. OF MERCURY | AVERAGE EFFECT ON PULSE RATE, BEATS PER MINUTE |
|---|---|---|---|---|---|
| 16 | 33 | On right side | 4 | 0 | +1 |
| | | On left side | 3 | −47 | −60 |
| | | On left side immediately following evulsion of sinus nerve | 3 | −3 | 0 |
| 13 | 46 | On right side | 3 | 0 | −1 |
| | | On left side | 3 | −34 | −27 |
| | | On left side immediately following evulsion of sinus nerve | 3 | +2 | +3 |
| 15 | 63 | On right side | 3 | −3 | +1 |
| 17 | 92 | On right side | 2 | +1 | −2 |
| | | On left side | 2 | −27 | −46 |
| | | On left side immediately following evulsion of sinus nerve | 2 | 0 | 0 |
| 18 | 240 | On right side | 3 | 0 | −1 |
| | | On left side | 3 | 0 | −55 |
| | | On left side immediately following evulsion of sinus nerve | 4 | 0 | −3 |

* With each trial the pressure in the isolated carotid sinus was raised. The changes that are produced in the blood pressure and heart rate by increasing the pressure in the carotid sinus are expressed as the increase or decrease from the values recorded immediately preceding the stimulation.

up on the hemostat, the evulsion was completed. The carotid sinus and all structures in the immediate neighborhood of the carotid sinus were not disturbed.

At periods, varying from one to eight months, the sensitivity of the carotid sinus on the side of the evulsion was tested. The technic employed

in testing the sensitivity of the sinus was exactly similar to that described in the foregoing series of experiments. In four of the five animals, the response of the sinus on the opposite side was also tested as a control, before and immediately after evulsion of the sinus nerves.

The results of this series of experiments have been very uniform. Varying the pressure within the carotid sinus on the side of the evulsion had no effect on the rate of the heart or on blood pressure (table 3). On the opposite side, which was used as a control, raising the pressure in the carotid

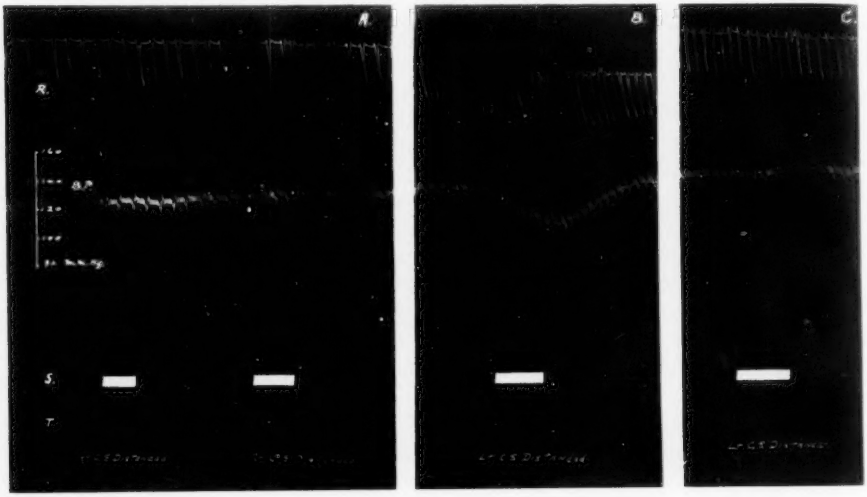


Fig. 3. Right carotid sinus nerve evulsed three months prior to the experiment. *R*, respiration, *B.P.*, blood pressure; *S*, signal; *T*, time in five second intervals. At signal, pressure is raised in isolated sinus and then immediately lowered. A, pressure raised in right sinus with no effect on heart rate, blood pressure, or respiration. B, pressure raised in left control sinus. Heart rate and blood pressure diminished. Respiration slightly increased. C, pressure raised in left control sinus immediately after evulsion of left carotid sinus nerve.

sinus produced the typical fall in blood pressure and slowing of the rate of the heart (fig. 3). Finally, evulsion of the sinus nerve on the side that was used as a control abolished the sensitivity of the carotid sinus to pressure. We may conclude that in the dog the cardiovascular components of the sinus reflex are conducted solely by the sinus nerve.

Comments. Denervation of the carotid sinus has been accomplished most frequently by one of two methods: 1, the carotid sinus is removed between ligatures, or 2, the structures between the bifurcation of the common carotid artery are ligated, detached from the blood vessels, and finally,

the region of the carotid sinus is painted with phenol, and then with alcohol (1), (7), (12), (13), (17).

We have developed a surgical approach to the sinus nerve which has been successfully carried out in more than fifty operations on the carotid sinus. In our experiments, evulsion of the sinus nerve alone removed the regulatory influence of the carotid sinus on the heart rate and on the blood pressure. The procedure therefore offers a method of denervation of the carotid sinus which does not impair the functions of other nerves in the region or the blood supply to the head.

## CONCLUSIONS

A study has been made of the anatomy of the carotid sinus region of the dog in order to determine the nerves ending in the carotid sinus. The physiologic function of the nerves supplying the sinus has been investigated and the following results obtained.

1. Anatomically, we have found that the carotid sinus possesses three sources of nerve supply: a branch from the posterior division of the glossopharyngeal nerve, a branch from the superior cervical ganglion, and a small nerve which accompanies the internal carotid artery.

2. Electric stimulation indicates that the nerve accompanying the internal carotid artery is not concerned in the carotid sinus reflex.

3. A study of the comparative responses of the sympathetic nerve and the sinus nerve to electric stimulation clearly indicates the greater importance of the sinus nerve.

4. On the basis of acute and survival denervation experiments it is concluded that the cardiovascular components of the sinus reflex are conducted solely through the sinus nerve.

5. Evulsion of the sinus nerve is submitted as a method of carotid sinus denervation and the surgical approach to the sinus nerve is described.

6. Our experiments lend support to the view that the carotid sinus operates entirely through a depressor mechanism.

## REFERENCES

(1) Cromer, S. P. and A. C. Ivy. Proc. Soc. Exper. Biol. and Med. **28:** 565, 1931.
(2) De Castro, F. Trav. du Lab. de Rech. biol. **24:** 365, 1926.
(3) De Castro, F. Trav. du Lab. de Rech. biol. **25:** 331, 1928.
(4) Daniélopolu, D., A. Aslan, I. Marcu, G. G. Proca and E. Manesco. Presse méd. **2:** 1585, 1927.
(5) Daniélopolu, D., I. Marcu, G. G. Proca and A. Aslan. Presse méd. **1:** 489, 1932.
(6) Garard, M. W. and P. R. Billingsly. Anat. Rec. **25:** 391, 1925.
(7) Green, M. F., A. F. Degroat and C. H. McDonald. This Journal **110:** 513, 1935.
(8) Hering, H. E. Münch. med. Wchnschr. **1:** 701, 1924.
(9) Hering, H. E. Münch. med. Wchnschr. **2:** 1265, 1775, 1924.

(10) HERING, H. E. Die Karotissinusreflexe auf Herz und Gefässe. Dresden, Th. Steinkopff, 1927.
(11) HEYMANS, C. AND J. J. BOUCKAERT. Compt. rend. Soc. de biol. **103:** 1930 1930.
(12) HEYMANS, C., J. J. BOUCKAERT AND L. DAUTREBANDE. Arch. Internat. de Pharmacodyn. et de Therap. **39:** 400, 1930.
(13) HEYMANS, C., J. J. BOUCKAERT AND P. REGNIERS. Le sinus carotidien. G. Doin and Company, Paris, 1933.
(14) KAHN, R. H. Ztschr. f. d. ges. exp. Med. **68:** 201, 1929.
(15) KOCH, E. Ztschr. f. Kreislaufforsch. **21:** 586, 1929.
(16) MOISSEJEFF, E. Ztschr. f. d. gesammt. exp. Med. **53:** 696, 1927.
(17) SCHMIDT, C. F. This Journal **102:** 94, 119, 1932.
(18) TOURNADE, A. AND MALMÉJAC. Compt. rend. Soc. de biol. **103:** 672, 1930.
(19) WRIGHT, S. Brit. Med. J. **1:** 457, 1932.

# ALTERATIONS OF RESPIRATORY MOVEMENTS INDUCED BY ELECTRICAL STIMULATION OF THE CEREBRAL CORTEX OF THE DOG[1]

WILBUR K. SMITH

*From the Department of Anatomy, The University of Rochester, School of Medicine and Dentistry, Rochester, New York*

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Several investigators have previously reported changes in the character of the normal reflex respiratory movements upon electrical stimulation of the cerebral cortex, but their results have been so diversified, both as to the respiratory changes induced and as to the cortical areas concerned, that no definite conclusions can be reached regarding either of these questions.

The work here reported reveals the presence of definite cortical areas which upon electrical stimulation evoke specific alterations in the respiratory movements.

METHOD. The dogs used in these experiments were anesthetized with ether to a depth just sufficient to prevent voluntary movements. In order to obviate respiratory arrest from closure of the larynx, the anesthetic was administered by a thick-walled rubber tube introduced through the mouth into the trachea. Kymographic records of the respirations were made by means of a Marey tambour connected to a pneumograph placed around the lower part of the thorax.

The cortex was exposed, in the manner described in detail in a previous report (Smith, 1935) as far medially as the longitudinal fissure, as far laterally and ventrally as the olfactory tracts, as far rostrally as the olfactory bulb, and as far caudally as the edge of the hemisphere. In order to effect such a complete exposure it is necessary to remove the bone forming the walls of the orbital cavity and of the frontal sinuses. The evacuation of the contents of the eyeball through a corneal incision expedites this procedure.

Stimulation of the exposed cortex was carried out by the unipolar method employing, as the current source, 60 cycle sine wave alternating current up to 3 volts with a current strength up to 0.8 milliampere. The stigmatic electrode was made of 24 gauge platinum wire, insulated except at the tip

[1] Presented at the Second International Neurological Congress, London, England, July 30, 1935.

and mounted on a fine spring of copper wire; the indifferent electrode consisted of a copper plate placed beneath the skin in the sacral region. During stimulation care was taken to apply the stimulating electrode perpendicular to the surface of the brain, thus keeping the area of contact as constant as possible.

The areas from which respiratory changes were elicited were plotted on a tracing of the exposed cortical surface made upon a piece of transparent celluloid sheeting, the tracing including both the blood vessels and the sulci. Upon completion of the experiment the blood in the vessels of the cortical surface was fixed by the application of formalin and the vascular pattern thus preserved. With the aid of the tracing, the position and extent of the areas from which respiratory changes were obtained could be easily identified in the brain after it had been removed from the animal.

For histological study blocks of the cortex were removed, dehydrated in graded alcohols, embedded in paraffin, sectioned at 20 microns and stained with thionin. All sections were cut as nearly as possible in a direction perpendicular to the cortical surface.

Results. In the cerebral cortex of the dog three separate and distinct cortical areas were found, each of which upon electrical excitation evoked a pronounced change in the respiratory movements.

Stimulation of an area in the lateral portion of the anterior sigmoid gyrus (fig. 1, *a*) produced an apparently immediate response in which the respiratory rate was markedly accelerated either with or without a slight decrease in the amplitude of the respiratory movements (fig. 2, a). The acceleration continued throughout the duration of the stimulation and ceased when the stimulating current was interrupted. The response, both from direct observation and from kymographic records, appeared to be a simple acceleration of the respiratory rate, characterized by a decrease in the duration of the expiratory pause. With a current source of 3 volts, acceleration of the rate was often obtained from a considerable portion of the anterior sigmoid gyrus, but as the voltage was reduced the responsive area became smaller. By thus gradually reducing the strength of the stimulating current, the most excitable area was found to be situated adjacent to the sulcus praesylvius in the rostral and lateral part of the anterior sigmoid gyrus. From the lateral portion of this area contractions of the neck muscles, in such a manner as to cause turning of the head toward the contralateral side, often occurred simultaneously with the alterations in respiration. In the medial part of the area nothing was observed except the changes in respiratory movements.

The amount of acceleration was found to depend upon several factors. In general it appeared to vary directly in proportion to the excitability of the cortex and the strength of the exciting current and inversely as regards the respiratory rate preceding the stimulation. The acceleration

was most pronounced during the first few seconds of the period of stimulalation and became less marked as the duration of the excitation increased. The acceleratory area was found to be present in apparently identical positions on the two sides of the brain. Bilateral simultaneous stimulation

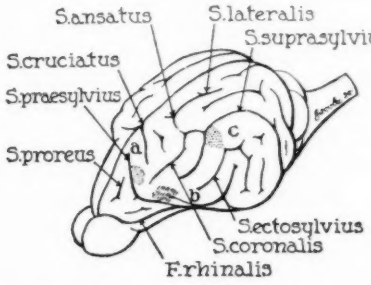


Fig. 1. Outline drawing of the left cerebral hemisphere of the dog showing the position of the cortical respiratory areas. *a*. Anterior sigmoid gyrus. *b*. Anterior composite gyrus. *c*. Ectosylvian gyrus.

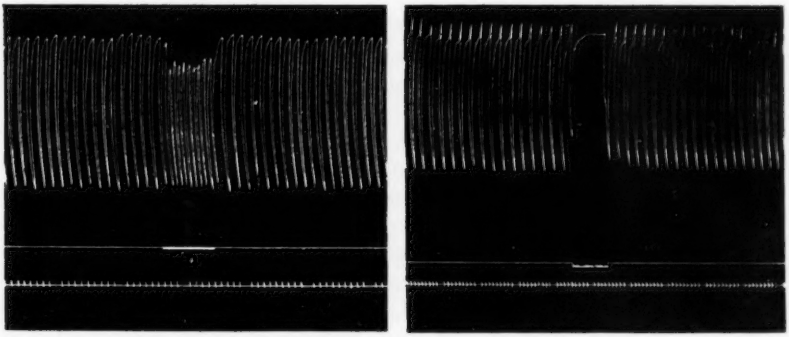


Fig. 2. *a*. Increase in the rate of the respiratory movements resulting from stimulation of the acceleratory area in the anterior sigmoid gyrus. *b*. Arrest of the respiratory movements with the thorax in the expiratory position resulting from stimulation of the inhibitory area in the anterior composite gyrus. The time is indicated in seconds. Inspiration is indicated by the downstroke; expiration by the upstroke.

of the acceleratory areas had a more pronounced effect than unilateral excitation.

Stimulation of an area in the rostral part of the gyrus compositus anterior (fig. 1, *b*) produced an effect upon the respiration entirely opposite to that described above. Excitation of this region had an inhibitory effect on the respiratory movements. Under favorable experimental conditions

with a stimulation of a few seconds' duration complete cessation of the respiratory movements occurred almost immediately, the arrest continuing throughout the duration of the excitation (fig. 2, *b*). This arrest was always found to occur with the thorax in the position of expiration. Moreover, in whatever phase of expiration or inspiration the stimulating current was applied the expiratory position of the thorax was immediately assumed and maintained throughout the period of arrest. That the arrest however cannot be maintained indefinitely is shown by the fact that after several seconds of complete respiratory cessation the movements again begin, at first shallow but gradually increasing in rate and amplitude so as to approach, but never attain, the rate which existed prior to the application of the stimulus. If the excitability of the cortex became lowered or if a weaker exciting current was used, then upon application of the stimulus there ensued a slowing of the respiratory rate characterized by a decrease in the amplitude of the respiratory excursions with the occurrence of shallow respirations. Expiration remained just as complete as it was preceding the stimulation. With a still weaker current one could elicit a slowing of respiration with or without a slight change in amplitude, and this altered respiration could be maintained for many seconds.

With a current source of 3 volts for the stimulating current some inhibitory effect was usually produced upon the respiratory movements from a considerable portion of the gyrus compositus anterior and the anterior part of the ectosylvian gyrus. The response, however, was most easily elicited from the rostral portion of the anterior composite gyrus as shown in figure 1. From this area an inhibitory effect could be elicited with a current source of 1 volt. With a current source of 3 volts swallowing movements and movements of the tongue could sometimes be elicited. If the strength of the current was considerably decreased the swallowing movements were not obtained, but the inhibitory effect on the respiratory movements remained.

Another area from which inhibitory respiratory effects were regularly obtained was found on the ectosylvian gyrus (fig. 1, *c*) at the position indicated in the figure. Stimulation of this area with a current source of 3 volts always was found to cause a pronounced slowing of the respiratory rate without much change in the amplitude of the respiratory excursions. Successive repeated stimulations of this area and the inhibitory area in the anterior composite gyrus, using the same strength of current source for all, indicates that the inhibitory effect from the area on the ectosylvian gyrus is less pronounced than that from the anterior composite gyrus.

The inhibitory areas, like the acceleratory area, were found to occupy apparently corresponding positions on the two sides of the brain. Simultaneous bilateral stimulation of the inhibitory areas was found to enhance considerably the degree of respiratory inhibition.

The acceleratory and inhibitory areas apparently give origin to cerebral cortical efferent fibers which pass to subcortical structures. This is evidenced by the finding that after removal of the responsive cortical area excitation of the underlying fibers produces results similar to those previously obtained upon stimulation of the cortex.

Bilateral section of both vago-sympathetic trunks and both phrenic nerves did not seem to affect the fundamental alterations of respiration elicited by stimulation of the acceleratory or inhibitory areas. Moreover, the relationship between the degree of response elicited by successive stimulations of the inhibitory area in the anterior composite gyrus and that elicited by stimulation of the inhibitory area in the ectosylvian gyrus remained unchanged.

Histological study of the cortical area from which acceleration was obtained showed that it was composed of two cytoarchitecturally different types of cortex. The medial part of the area was found to belong to the precentral agranular frontal type of cortex which has been numerically designated by Brodmann as constituting area 6. This cortical area is characterized by poor stratification and by the absence of granular layers. Toward the lateral part of the area this cortex changes over into the precentral agranular gigantopyramidal type (area 4 of Brodmann), the characteristic features of which are its poor lamination, the absence of granular layers and the presence of large and highly chromophilic cells in layer V.

The most rostral part of the inhibitory area in the anterior composite gyrus possesses a structure which places it, like the acceleratory area, in the precentral agranular frontal type (area 6). However, the cortex of this inhibitory area is not identical with the cortex of the medial part of the acceleratory area, but differs from it chiefly in its greater richness of cells and, although granular layers are not well defined, in the presence of a greater number of granule cells. Caudally, the cortex of the inhibitory area represents a transition between the precentral agranular frontal type and the more caudally situated granular type which possesses definite granular layers.

The inhibitory area in the ectosylvian gyrus is composed of six-layered granular cortex with well defined internal and external granular layers. It lies within the cortical area considered by Brodmann to belong to the temporal field and numerically designated by him as area 50.

COMMENT. To attempt to account for or to explain the highly inconstant and widely divergent results obtained by previous investigators would be a futile task, but in many cases, among other things, the use of unanesthetized animals, the use of anesthetics such as morphia and chloral hydrate which unduly depress the excitability of the cortex, the use of too strong currents for stimulation, and undue loss of blood seem to have vitiated the results obtained. In addition, none of the previous investi-

gators except Spencer (1894) appear to have eliminated the possibility of respiratory changes occurring from closure of the larynx as a result of stimulation of the cortical motor centers for the laryngeal muscles.

The fact that alterations of respiration were never obtained from excitation of that part of the cerebral cortex which lies rostral to the sulcus praesylvius, even with a current strength considerably in excess of that furnished by three volts, or with strong faradic stimulation, is in direct contradiction to the findings of Ferrier (1876), Munk (1882), Gianelli (1900) and Polimanti (1906), all of whom reported alterations in the respiratory movements upon stimulation of a part or all of this zone.

In addition, the evidence adduced from my experiments refutes the contentions of Bochefontaine (1876) and Francois-Franck (1888) that only excitation of the so-called "motor zone," by which they probably mean the sigmoid gyri, induces changes in respiratory movements and that no relation exists between the cortical area excited and the resultant change which ensues. The inhibitory phenomena cannot be due to tetanic contraction of the diaphragm, as Munk (1882) concluded from his experiments, since it can be obtained after bilateral section of the phrenic nerves.

## SUMMARY AND CONCLUSIONS

The results obtained in this investigation lead to the conclusion that there are present in the cerebral cortex of the dog definite areas which when electrically stimulated effect specific alterations in the respiratory movements.

Stimulation of an area in the lateral part of the anterior sigmoid gyrus was found to produce a marked acceleration of the respiratory rate.

Stimulation of an area in the rostral part of the anterior composite gyrus caused either arrest of respiration with the thorax in the expiratory position or a marked slowing of the respiratory rate with the occurrence of shallow respirations.

Stimulation of an area in the ectosylvian gyrus also produced an inhibitory response, but the effect was less pronounced than that obtained from the anterior composite gyrus.

The acceleratory and inhibitory areas were found to be present in both cerebral hemispheres, and bilateral stimulation was found to have a greater effect than unilateral stimulation.

Cytoarchitecturally, the acceleratory area is composed of two types of cortex, the precentral agranular frontal type (area 6 of Brodmann) in its medial part and the precentral agranular gigantopyramidal type (area 4) in its lateral part.

The cortex of the rostral part of the inhibitory area in the anterior composite gyrus belongs to the precentral agranular frontal type (area 6); the caudal portion appears to be a transition zone between area 6 and the more

caudal granular cortex. The cortex of this portion of area 6 differs in some details from that part of area 6 in the acceleratory area.

The inhibitory area in the ectosylvian gyrus was found to be composed of a six-layered granular cortex of a type considered by Brodmann as constituting a part of the temporal field and numerically designated by him as area 50.

Bilateral section of the vago-sympathetic trunks and the phrenic nerves did not appear to alter the response obtained upon stimulation of the cortical respiratory areas. Therefore, it may be concluded that the phrenic and vago-sympathetic nerves are not essential pathways in the production of either response. It seems likely that the efferent cortical impulses exercise an influence, either directly or indirectly, over the nervous respiratory mechanism situated in the medulla oblongata, and thus affect the respiratory muscles as a functional unit.

## REFERENCES

BOCHEFONTAINE, L. Arch. de Physiol. **3:** 140, 1876.

FERRIER, D. The functions of the brain. New York, G. P. Putnam's Sons, ed. 1, 1876; ed. 2, 1886.

FRANCOIS-FRANCK, C. Mem. Soc. de Biol. **40:** 27, 1888.

GIANELLI, A. Ann. di Neurol. **17:** 442, 1900.

MUNK, H. Sitzunsb. d. k. Preuss. Akad. d. Wissensch. zu Berlin, 1882, p. 753.

POLIMANTI, O. Contributo alla fisiologia ed all'anatomia die lobi frontali. G. Bertero, Rome, 1906.

SMITH, W. K. J. Comp. Neurol. **62:** 421, 1935.

SPENCER, W. G. Philosoph. Trans. Roy. Soc. London, Series B, **185:** 609, 1894.

# THE CARDIAC OUTPUT IN THE STANDING POSITION

JOHN C. SCOTT

*From the Department of Physiology of Hahnemann Medical College of Philadelphia, Philadelphia, Penna.*

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The effect of posture upon cardiac output has been discussed recently by many workers but the opinions expressed differ widely even among those that utilize the same method. Donal, Gamble and Shaw (1934) find a decrease in cardiac output on standing as estimated by the modified ethyl iodide method, a result which is supported by Schneider and Crampton (1934) from estimates by Grollman's acetylene procedure. Yet Grollman himself (1928) claimed that the cardiac outputs were identical in the lying and standing positions. However, quite recently Nylin (1934) has pointed out that Grollman's own figures show a small average reduction (0.20 liter) in output with standing, which is twice the mean error. Bazett and his co-workers (1935) found evidence of seasonal variations in the pulse rate and blood pressure changes induced by standing; they found some variation, possibly seasonal in origin, in the effect of standing on cardiac output even in the same subject, but using the acetylene method confirmed Grollman for the most part.

Consequently a large series of determinations have been made on a single subject in the recumbent, sitting and standing positions and these observations have extended over a year to exclude possible seasonal variations. The number of observations total 129, of which 21 were obtained in the recumbent position, 56 in the sitting position and 52 in the standing position. The data on this subject are therefore adequate for statistical treatment, while most of the deductions made in the literature have on the contrary been founded on data which have been both variable and scanty.

METHOD. The Grollman acetylene method was used on a normal adult male of 1.70 square meters surface area (subject 8 of the data reported by Bazett et al.). The precautions suggested by Grollman (1928) were taken in the determination of the cardiac output in the recumbent position. The observations were made immediately upon awakening in the morning with the exception of some of the sitting experiments which were done about one hour after arising. All experiments were done under basal conditions. Measurement of oxygen consumption by a Sanborn apparatus was followed by the acetylene rebreathing procedure with sam-

ples taken at 14 and 20 seconds. In observations during standing the whole procedure lasted some ten minutes. Duplicate analyses of the samples were always made and agreed usually within 0.02 per cent or less. In absorbing $CO_2$ a slight variation in technic was used to compensate for the absorption of acetylene by the $CO_2$ absorber. A 20 per cent KOH solution saturated with NaCl was used and a standard movement of three slow swings was found to remove all $CO_2$; the amount of acetylene removed by the same movements was also determined and the apparent percentages were corrected accordingly.

The subject was careful to remain erect but comfortably relaxed, leaning against the wall, but in a few experiments this procedure was varied and he intentionally made slight movements; however such variations in the procedure appeared to have no significant effect on the cardiac output.

Results. The values obtained with the subject standing are given in table 1; the average values obtained on the subject recumbent, sitting and standing are indicated in table 2. While these data show quite definitely that there is usually a reduction in cardiac output on standing, that this reduction is far beyond the probable error of the method and must be significant, the values themselves demonstrate a considerable variability. This variability is present also in the oxygen consumption figures although to a lesser extent. The analysis of the figures in two groups, one involving all data obtained between June 10 and August 28 when the weather was mostly warm and the other data obtained between November 28 and April 3 when the outdoor weather was cold (but the indoor temperature not very different from that of the first set) does not appear at first glance to clarify the issue since the differences between the means of the cardiac indices of the two groups does not exceed the probable error of either mean. However if the individual arterio-venous oxygen differences are compared with the oxygen consumption observations on the same occasion a very great difference is seen in the two groups. In the winter group (fig. 1) high AV differences are associated with high oxygen consumption and the high values fall definitely along a line with the exception of only 5 of the 29 observations. On the other hand in the summer group high AV differences are associated with low oxygen consumptions and again the values (with the exception of 4 out of a total of 23) fall along a line with a slope quite the opposite of that representing the winter values. The conditions are obviously quite different and though similar cardiac outputs are observed, the physiological adjustments necessary to attain them must have been quite different.

If the cardiac indices similarly be plotted against the oxygen consumption, the cardiac outputs increase with high oxygen consumptions but more so in the summer than in the winter months. At low oxygen consumptions cardiac outputs are low in the summer months; in the winter

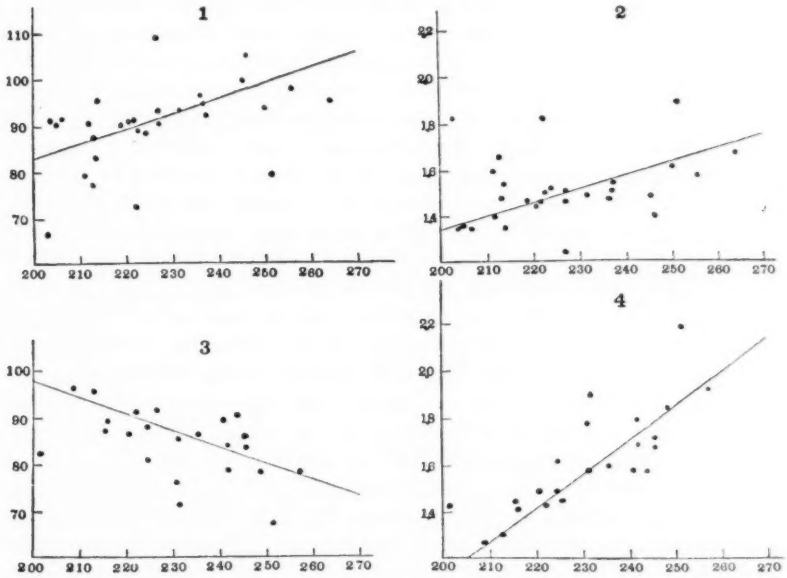
TABLE 1

| NO. | DATE | OXYGEN | A.V.D. | C.O. | C.I. |
|---|---|---|---|---|---|
| 1 | 11/28/34 | 226.5 | 109.00 | 2.06 | 1.24 |
| 2 | 12/ 3/34 | 236.0 | 96.60 | 2.44 | 1.47 |
| 3 | 12/ 4/34 | 237.0 | 92.64 | 2.56 | 1.54 |
| 4 | 12/ 5/34 | 250.0 | 93.65 | 2.67 | 1.61 |
| 5 | 12/ 6/34 | 211.8 | 90.95 | 2.33 | 1.40 |
| 6 | 12/10/34 | 255.8 | 98.00 | 2.61 | 1.57 |
| 7 | 12/11/34 | 245.1 | 99.82 | 2.45 | 1.48 |
| 8 | 12/12/34 | 246.0 | 105.20 | 2.33 | 1.40 |
| 9 | 12/13/34 | 264.0 | 95.15 | 2.77 | 1.67 |
| 10 | 12/18/34 | 251.2 | 79.74 | 3.15 | 1.89 |
| 11 | 1/ 9/35 | 223.8 | 88.61 | 2.52 | 1.52 |
| 12 | 1/10/35 | 226.8 | 90.91 | 2.51 | 1.51 |
| 13 | 1/14/35 | 213.8 | 95.08 | 2.25 | 1.35 |
| 14 | 1/15/35 | 222.0 | 72.54 | 3.05 | 1.83 |
| 15 | 1/16/35 | 213.0 | 87.80 | 2.46 | 1.48 |
| 16 | 1/17/35 | 222.4 | 89.24 | 2.49 | 1.50 |
| 17 | 1/23/35 | 202.7 | 66.47 | 3.05 | 1.83 |
| 18 | 1/28/35 | 231.4 | 93.43 | 2.47 | 1.49 |
| 19 | 1/29/35 | 236.7 | 94.60 | 2.50 | 1.50 |
| 20 | 1/30/35 | 203.7 | 91.16 | 2.23 | 1.34 |
| 21 | 1/31/35 | 205.0 | 90.60 | 2.26 | 1.36 |
| 22 | 2/ 4/35 | 212.8 | 77.25 | 2.75 | 1.66 |
| 23 | 2/ 5/35 | 211.3 | 79.30 | 2.67 | 1.60 |
| 24 | 2/ 7/35 | 221.4 | 91.50 | 2.43 | 1.46 |
| 25 | 2/21/35 | 220.6 | 91.26 | 2.42 | 1.45 |
| 26 | 2/25/35 | 213.6 | 83.43 | 2.56 | 1.54 |
| 27 | 3/14/35 | 218.8 | 90.03 | 2.43 | 1.46 |
| 28 | 4/ 2/35 | 206.4 | 91.77 | 2.25 | 1.35 |
| 29 | 4/ 3/35 | 226.8 | 93.71 | 2.42 | 1.46 |
| 30 | 6/10/35 | 215.9 | 89.50 | 2.41 | 1.42 |
| 31 | 6/11/35 | 240.6 | 89.66 | 2.68 | 1.58 |
| 32 | 6/12/35 | 231.4 | 71.68 | 3.23 | 1.90 |
| 33 | 6/13/35 | 257.2 | 78.50 | 3.28 | 1.93 |
| 34 | 7/12/35 | 235.1 | 86.52 | 2.72 | 1.60 |
| 35 | 7/13/35 | 201.5 | 82.60 | 2.44 | 1.43 |
| 36 | 7/18/35 | 245.5 | 83.91 | 2.93 | 1.72 |
| 37 | 7/20/35 | 224.5 | 88.37 | 2.54 | 1.49 |
| 38 | 7/22/35 | 241.7 | 84.02 | 2.88 | 1.69 |
| 39 | 7/23/35 | 230.8 | 76.10 | 3.03 | 1.78 |
| 40 | 7/24/35 | 245.8 | 86.12 | 2.85 | 1.68 |
| 41 | 7/25/35 | 241.7 | 78.81 | 3.07 | 1.80 |
| 42 | 7/27/35 | 224.2 | 81.47 | 2.75 | 1.62 |
| 43 | 7/30/35 | 251.1 | 67.33 | 3.73 | 2.19 |
| 44 | 7/31/35 | 243.8 | 90.60 | 2.69 | 1.58 |
| 45 | 8/ 8/35 | 248.5 | 78.72 | 3.15 | 1.85 |
| 46 | 8/ 9/35 | 231.0 | 85.96 | 2.69 | 1.58 |
| 47 | 8/13/35 | 221.8 | 91.10 | 2.43 | 1.43 |
| 48 | 8/15/35 | 208.6 | 96.52 | 2.16 | 1.27 |
| 49 | 8/16/35 | 212.7 | 95.96 | 2.22 | 1.30 |
| 50 | 8/20/35 | 226.3 | 91.81 | 2.46 | 1.45 |
| 51 | 8/27/35 | 215.4 | 87.22 | 2.47 | 1.45 |
| 52 | 8/28/35 | 220.5 | 86.86 | 2.54 | 1.49 |
| Average. . . . . | | 228.3 | 87.86 | 2.62 | 1.56 |

either low or high values may be found. Similar treatment of data for the recumbent and sitting positions obtained mainly in the winter shows no such relationships.

TABLE 2

| POSITION, NO. OF EXP. | OXYGEN CONSUMPTION (CC. PER MIN.) | | | AV. DIFFERENCE (CC. PER LITER) | | | CARDIAC INDEX | | | STAND. DEV. |
|---|---|---|---|---|---|---|---|---|---|---|
| | Av. | Max. | Min | Av. | Max. | Min. | Av. | Max. | Min | |
| Standing, Summer (23) | 231 | 257 | 202 | 84.75 | 96.50 | 67.30 | 1.62 | 2.19 | 1.27 | ±0.22 |
| Standing, Winter (29) | 226 | 264 | 203 | 90.32 | 109.00 | 66.50 | 1.51 | 1.89 | 1.24 | ±0.15 |
| Total (52) | 228 | 264 | 202 | 87.86 | 109.00 | 66.50 | 1.56 | 2.19 | 1.24 | |
| Sitting (56) | 201 | 224 | 183 | 60.91 | 82.05 | 47.07 | 1.99 | 2.39 | 1.47 | ±0.17 |
| Lying (21) | 207 | 263 | 193 | 59.97 | 70.03 | 52.38 | 2.10 | 2.38 | 1.85 | ±0.16 |




Figs. 1 and 3 show the relationship of arterio-venous oxygen difference (cc. per liter) to oxygen consumption (cc. per minute); figures 2 and 4 show the cardiac indices plotted also against oxygen consumption. Figures 1 and 2 represent data obtained in the winter, figures 3 and 4, summer data. In each case oxygen consumption is represented as abscissae.

Discussion. The data of figures 1 to 4 demonstrate that the main cause of the variations must be physiological and not experimental, for it is inconceivable that otherwise there could be the demonstrated relation-

ship between oxygen consumption and AV difference, since these two values are measured by quite independent procedures. Even the few values, which do not fit the curves, are probably in many cases dependent on physiological variants; for instance the data of table 1 show that similar discrepancies are often found on consecutive days. Thus the high cardiac output of June 12, which is above the minimum recumbent value is presumably valid, since it was duplicated on the following day; yet high outputs are not necessarily associated with summer conditions for the low value of August 15 is equally well established by the duplication of the figures in every detail on August 16. Though a few of the discrepant data may depend on experimental error for the most part they must be assumed correct and Grollman's technical method appears to be adequate to give reproducible figures when the subject is basal, if variations in the environmental conditions can be excluded. Attempts to correlate the changes with the room temperature have failed. It is probably impossible to investigate the factors involved with the control available in an ordinary room.

Whatever the factors involved may be, they appear to be accelerated and rendered easier to investigate by the process of standing, since the variations in cardiac output in the recumbent and sitting positions are of similar magnitude to those obtained with the subject standing at the same time of the year, yet no correlation between oxygen consumption and AV difference is demonstrable. Certainly the subject on getting out of bed and standing up exposes himself to an environmental temperature which may, or may not, be considerably below that to which the body has been previously exposed. When the present series of experiments were undertaken it seemed probable that reduction of cardiac output on standing as the result of gravity effects might be maximal if the experiments were performed immediately upon wakening. The data show a reduction on standing fully equivalent to that described by others, in spite of the fact that the thermal stimulation may otherwise have tended to introduce an opposing factor. The data cannot reasonably be expected to agree with that obtained by Bazett et al. even on the same subject since in these experiments the conditions were quite different. The subjects were either partially or completely nude; in many cases the room was so warm that the subject was sweating while lying down, and noted a definite increase in comfort on standing up when a larger surface was available for evaporation. The experiments were conducted later in the day, and the subject leaned forward in a sling instead of resting against the wall.

While the complexities prevent an analysis of the factors involved in causing variations in cardiac output, yet the opposite correlation of AV differences with oxygen consumption in the summer and winter can be

readily explained on general principles. When the oxygen consumption is high in the winter it is likely to be associated with a metabolic response to cold, and consequently with an increased blood supply to the muscles. One would therefore anticipate the correlation of a high oxygen consumption with a high AV difference, a correlation which is observed. On the contrary in the summer a high oxygen consumption is likely to be associated with a high average body temperature, with the metabolism increased as a consequence of the temperature coefficients of chemical reactions; under these conditions increased efficiency of heat loss would be essential and require an increased circulation through the skin with its low metabolism so that a high oxygen consumption should be associated with a lowering of the AV difference. This is the case. The principles involved are particularly well demonstrated in the values both for heat production and heat loss obtained by Houghten et al. (1929). The data are then explicable on this basis if other factors are assumed to complicate the issue. For instance in the winter the cardiac outputs appear to be particularly variable when the oxygen consumption is low; if the low oxygen consumption depends on a low mean body temperature without either reflex or central stimulation of the metabolic reaction to cold the external conditions might still be such as to give either a marked vasoconstriction of the skin or no such reaction; it is unlikely that the effect on cardiac output would be identical in the two cases.

Finally a plea may be made for a more careful standardization of the conditions of experimentation. Though the condition of the subject on awakening is probably more nearly standard, more truly basal, than at later times in the day, it is not constant enough to remove all physiological variations. Probably a prolonged stay in an air conditioned room would be essential, and even then the possible existence of seasonal factors would have to be investigated.

## SUMMARY

The cardiac output of a single individual has been observed in the recumbent, sitting and standing positions. During the winter season in the standing position high AV differences are usually associated with high oxygen consumption; in the summer this relationship is reversed. The average standing cardiac output is lower than that of the recumbent or sitting positions. The output is influenced by various undefined environmental factors under so called basal conditions particularly if the subject is standing.

## REFERENCES

Bazett, H. C., F. H. Cotton, L. B. Laplace and J. C. Scott. This Journal **113**: 312, 1935.

DONAL, J. S., C. J. GAMBLE AND R. SHAW. This Journal **109:** 666, 1934.
GRIFFITH, F. R., G. W. PUSCHER, K. A. BROWNELL AND J. D. KLEIN. This Journal **88:** 295, 1929.
GROLLMAN, A. This Journal **86:** 285, 1928.
The cardiac output of man in health and disease. Charles C. Thomas, Springfield, Ill., 1932.
HOUGHTEN, F. C., W. W. TEAGUE, W. E. MILLER AND J. D. KLEIN. This Journal **88:** 386, 1929.
NYLIN, G. Skandinav. Arch. f. Physiol. **69:** 1934.
SCHNEIDER, E. C. AND C. B. CRAMPTON. This Journal **110:** 14, 1934.

# THE ACTION OF THE PREPARATIONS FROM THE POSTERIOR LOBE OF THE PITUITARY GLAND UPON THE IMBIBITION OF WATER BY FROGS

FRANCES K. OLDHAM

*From the Department of Pharmacology, McGill University, Montreal, Canada*

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During the past fifteen years several investigators have shown that extracts of the posterior lobe of the pituitary gland when injected into frogs give rise to a temporary increase in weight. The increases observed vary somewhat in magnitude and duration; this is probably due to seasonal variations in susceptibility, to the fact that different species have been employed, and to differences in the amount of the active substance injected. However, the general sequence of events consists of a gradual increase in weight, reaching a maximum usually in from one to five hours, followed by a more gradual return to the normal weight in from eight to twenty-four hours, though increases lasting for several days have been reported.

This phenomenon is of interest because of two problems which it raises, viz., 1, which constituent of posterior lobe extracts is the responsible agent—the pressor, oxytocic, melanophore, or some hitherto unknown constituent; 2, what is the physiological mechanism—change in skin permeability, in tissue hydration, or in renal function? This investigation is concerned with the former.

Brunn (1), on observing that pituglandol gave about twice as great an increase as did corresponding doses of two other preparations—hypophysin and pituitrin—suggested that the effect paralleled the intermediate lobe content. Unfortunately he gives no data concerning the relative intermediate lobe content of the three preparations. Bělehrádek and Huxley (2), who compared the effects of pituitrin on larval and metamorphosed Amblystoma, observed that the weight changes coincided with the melanophore changes. Steggerda (3) considered that the increase in weight might be due to an increased skin permeability brought about in some way by the expansion of the melanophores.

In 1930, Heller (4) studied the effects of pitressin and pitocin, and found that the oxytocic fraction caused a greater increase in weight than the pressor. He found also that the German preparations, tonephin and orasthin, are indistinguishable in action from pitressin and pitocin. The

results of such a comparison would be significant only if the methods of preparing these two substances were original, but on that point there appears to be nothing published. Steggerda (5) confirmed Heller's results and reported also that the activity of pituitrin was intermediate, a result which indicates that neither the pressor nor the oxytocic hormone is the active substance. Unfortunately, Steggerda expressed his doses in cubic centimeters instead of units, and as the preparations employed are put up in various strengths, one can only guess at the dosage in units which he used. Novelli (6), using toads, found the oxytocic substance to be only a third as effective as the pressor.

About two years ago, a new method of separating the pressor and oxytocic substances of the pituitary was developed in this laboratory (7). The pressor preparation was designated as postlobin-V, the oxytocic as postlobin-O. By studying the action of these substances in parallel with pitressin and pitocin, it was hoped that it would be possible to determine whether both the pressor substance and the oxytocic substance exert some action, or whether a substance which contaminates pressor and oxytocic preparations is the active agent, since, if such a substance exists, it might be distributed in different proportions in the two pressor and in the two oxytocic preparations. On the other hand, if no difference could be detected between the actions of the two pressor preparations and those of the two oxytocic preparations, the conclusion that both the pressor and oxytocic substances have an action of the kind in question would be strengthened.

A melanophore-dilating preparation which had very little pressor or oxytocic action was also studied.

METHOD. Since the injection of pituitary extract causes frogs to imbibe water, it is a reasonable inference that if saline is administered prior to, or along with, the pituitary extract, the elimination of the saline will be delayed. Consequently, two types of experiments were performed. In the first, 0.5 mil of saline containing the agent being studied was injected into the dorsal lymph sac of each of a series of frogs, and after each injection the animal (Rana pipiens) was weighed and placed in a numbered cage. The wire cages confining the animals rested upon a support about 1 cm. beneath the surface of the water in a large bath. At half-hour intervals the urine was expelled by pressure and the animals were dried with a towel and weighed. In the second type of experiment, the agent being studied was contained in 5 mil of saline. Injection and weighing were conducted as in the first type. In all cases the animals had been kept in the bath at room temperature during the night preceding the experiment.

Fifteen frogs were used for each experiment of the first type; controls in each case were found to be unnecessary. In the second type, six

animals received saline only and six the same quantity of saline containing the extract.

The average weight at each weighing was calculated from the individual weights, so that the changes could be expressed as the average increase or decrease in percentage of the weight immediately after the injection. In the graphs, the abscissas represent the time in hours, and the ordinates the change in weight per cent. All the frogs were females, except in the "A" experiment of figure 2, in which case half were males and half females. The weight increases induced by pituitary preparations are greater in the spring than in the autumn.

*Comparison of the activities of pitocin and postlobin-O.* It has been impossible to detect any difference between the activities of pitocin and postlobin-O. Figure 1 represents three experiments with each.

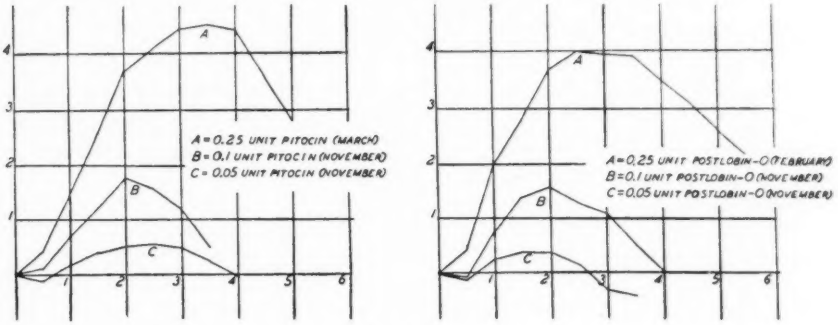


Fig. 1

*Comparison of the activities of pitressin and postlobin-V.* Neither has it been possible to differentiate between the activities of pitressin and postlobin-V, as an examination of figure 2 will show. The pair of experiments in which the dosage was 0.05 unit shows slight losses in weight, but this is less than is observed in control experiments with saline alone, which are also shown in figure 2. Inasmuch as the first weighings were made after the injections, a loss of weight is to be expected as a result of the elimination of the saline if no opposing element is involved.

*Comparison of the activities of oxytocic and pressor preparations.* The foregoing experiments show that the two oxytocic preparations have a stronger action than the pressor preparations. Some idea of the ratio of these actions may be gained by comparing figures 1 and 2. Obviously, the ratio is less than 5:1, since 0.05 unit of the oxytocic preparations exerted less action than five times as much of the pressor preparations. On the other hand, the ratio is greater than 2.5:1, since 0.1 unit of either oxytocic preparation caused a greater effect than 0.25 unit of either pressor

preparation. The *A* curves of these two figures are not comparable, because some of the experiments were performed in March when susceptibility is greater.

The obvious conclusion to be drawn is that both the oxytocic and the pressor substances exert the action in question, though to different degrees, or a third constituent contaminates both pitocin and postlobin-O to equal extents, and pitressin and postlobin-V to equal though lesser extents. The latter conclusion is less likely, though not impossible. That the oxytocic and pressor substances might differ only quantitatively as regards the action studied is rendered plausible on the basis of their apparently close chemical similarity (8).

*The activity of whole posterior lobe extract as compared with the activities of its pressor and oxytocic constituents.* Since whole posterior lobe extract contains both pressor and oxytocic constituents, one would expect it to

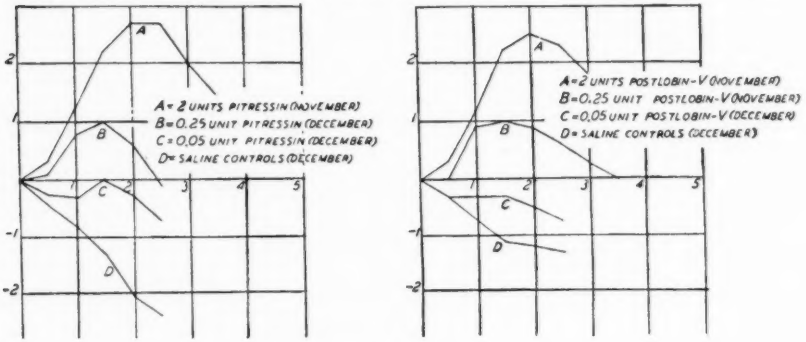


Fig. 2

be at least slightly more active than the oxytocic substance alone, and perhaps considerably more active, unless there is no loss of the hypothetical substance in the purification process, if a separate substance were involved. However, the results shown in figure 3 are hardly distinguishable from those obtained when the oxytocic substance alone was used (fig. 1). It may be concluded, therefore, from the similarity in action between whole pituitary extract and that of the oxytocic substance, that there is no evidence of a hitherto unknown constituent being responsible for the imbibition of water by frogs.

*Elimination of injected saline.* When the technic of the experiment was reversed and the influence upon the decrease in weight following the injection of saline was studied, the conclusions deducible were the same as in the imbibition experiments. Thus, figure 4 shows that 0.1 unit of pressor substance does not affect the elimination of saline, while 0.1 unit of oxytocic substance has a marked effect. This figure shows too that

0.1 unit of oxytocic substance exerts an effect which is slightly greater than that exerted by 0.4 of a pressor unit. It may be noted also that,

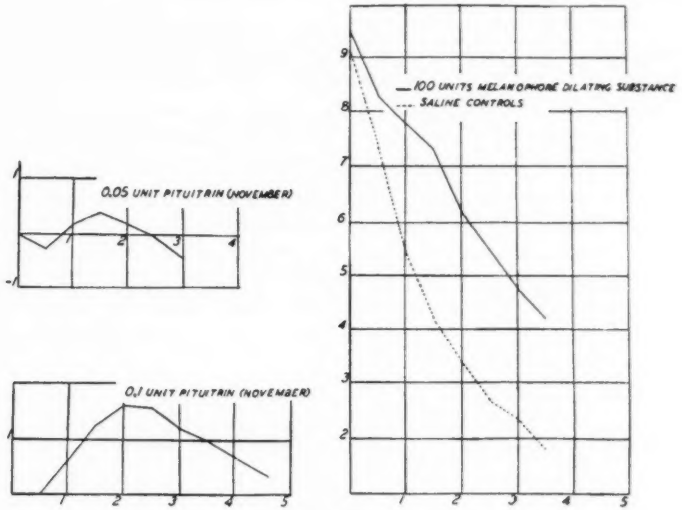


Fig. 3

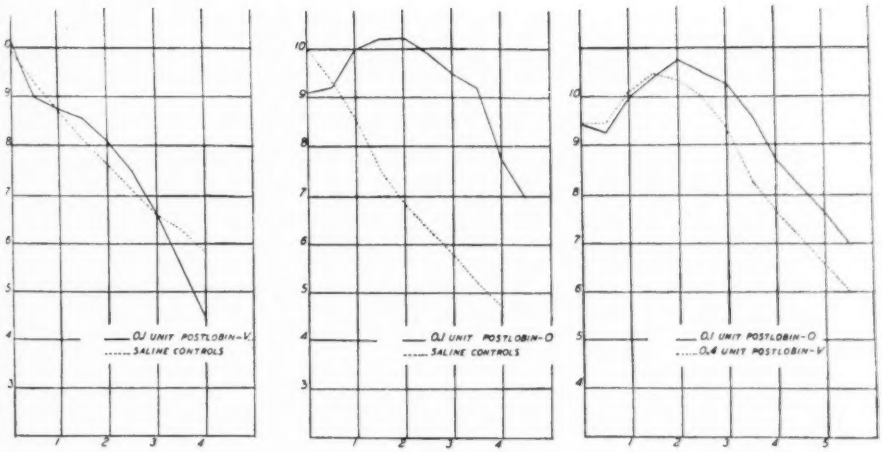


Fig. 4

whereas in the control experiment the weight begins to fall immediately following the injection of the saline, the oxytocic substance, and the pressor

too, in sufficiently high dosage, even under the conditions of this type of experiment, cause the weights to increase for a time, as in the simple imbibition experiments.

*The action of the melanophore hormone.* A preparation of the pituitary melanophore hormone, obtained from residues which were collected in the process of isolation of the pressor and oxytocic substances, was tried. (The method of preparation will be the subject of a future communication from this laboratory.) As assayed by comparison with standard pituitary powder, using the melanophore-dilating action upon frogs for the assay, this preparation was more than 100 times as active as the standard powder which was employed for comparison. It was almost devoid of pressor and oxytocic actions. Figure 3 shows that 0.5 mgm. of this preparation, equivalent in action to at least 100 units of our standard powder, exerted so little effect upon the elimination of the 5 mils of saline in which it was contained that it may be safely concluded that the melanophore hormone is not involved in the action studied.

REMARKS. While this study has been confined to an attempt to determine the constituent or constituents of the pituitary gland which affects the absorption and retention of water by frogs, attention should be called to the fact that, since the pressor constituent is the one which exerts the anti-diuretic action in mammals, it is doubtful whether there is any fundamental connection between these two actions. However, one might maintain with some degree of plausibility that in view of the chemical similarity of the pressor and oxytocic substances and the remote relationship of amphibians and mammals, differences in action are not surprising and do not prove necessarily that there is no connection between the two actions.

## SUMMARY

Both the oxytocic and the pressor hormone of the pituitary gland cause the imbibition of water by frogs. The potency of the oxytocic substance is between 2.5 and 5 times that of the pressor. The melanophore-dilating hormone is not involved in the action.

The author wishes to express appreciation to Professor Stehle for his helpful suggestions throughout the course of this work.

## REFERENCES

(1) BRUNN. Ztschr. f. d. ges. exper. Med. **25**: 171, 1921.
(2) BĚLEHRÁDEK AND HUXLEY. Brit. J. Exper. Biol. **5**: 89, 1927–28.
(3) STEGGERDA. This Journal **98**: 255, 1931.
(4) HELLER. Arch. f. exper. Path. u. Pharmakol. **157**: 323, 1930.
(5) STEGGERDA. Proc. Soc. Exper. Biol. and Med. **32**: 425, 1934.
(6) NOVELLI. Rev. Sci. Argentina Biol. **8**: 631, 1932.
(7) STEHLE. J. Biol. Chem. **102**: 573, 1933.
(8) STEHLE AND FRASER. J. Pharmacol. Exper. Therap. **55**: 136, 1935.

# THE INFLUENCE OF PREVIOUS FEEDING ON THE NITROGEN EXCRETION OF FASTING BIRDS

JAMES C. FRITZ, WALTER A. HENDRICKS AND HARRY W. TITUS

*From the Beltsville Research Center, United States Department of Agriculture, Beltsville, Maryland*

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Little work has been done on the fasting metabolism of poultry. A few studies on fasting birds have been reported by Shimanski (1879), Kuckein (1882), Voit (1901), Pashutin (1902), and Phillips, Ashworth, and Brody (1932). In many cases data obtained on one species of animal can be applied to another species without introducing serious errors, but data obtained on mammals may not be applicable to birds because there are important differences in anatomy and nitrogen metabolism.

Nitrogen excretion during fasting has been extensively studied in various mammals. In man and in the dog there is ordinarily a decrease in nitrogen excretion after food is withdrawn. In the former this is usually followed by a peak in nitrogen excretion on the second or third day of fasting (Benedict, 1907; Howe, Mattill and Hawk, 1911). Typical data for fasting dogs show a similar, but less pronounced, increase in nitrogen excretion between the second and fourth days (Morgulis, 1923). Morgulis attributes this transient decrease to the protein sparing action of carbohydrates present in the body. Benedict and Ritzman (1927), working with mature steers, noted that the nitrogen excretion usually increased on the first day of the fast, remained fairly constant for about three days, and then decreased rapidly.

According to Phillips, Ashworth and Brody (1932) the total nitrogen excretion of birds remains fairly constant, after an initial decrease following the withdrawal of feed, until the fast reaches an advanced stage.

The object of this study was threefold: first, to learn how the previous feeding influences the quantity of nitrogen excreted daily during a fast; second, to ascertain the manner in which the nitrogen excretion of mature birds changes after the withdrawal of feed; and third, to determine how long the fast must be continued in order for the daily nitrogen excretion to become practically constant.

MATERIALS AND METHODS. Twenty-seven Rhode Island Red cocks, aged twelve to eighteen months, were used in this experiment. All birds were in excellent health but not very fat. Prior to the time of the experiment these birds were kept on range.

The birds were divided into five groups and fed the diets shown in table 1. The analyses of these diets are shown in table 2. Diet 1 was a normal diet, designed to supply the needs of birds kept in strict confinement. Diets 2 and 3 were high- and low-protein diets, respectively, with all the protein from dried buttermilk. Diets 4 and 5 were high- and low-protein

TABLE 1

*Composition of the diets*

| INGREDIENT | DIET 1 | DIET 2 | DIET 3 | DIET 4 | DIET 5 |
|---|---|---|---|---|---|
| | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| Ground whole wheat | 28.000 | | | | |
| Ground yellow corn | 17.800 | | | | |
| Rolled oats | 15.000 | | | | |
| Desiccated meat meal | 7.750 | | | 27.780 | 6.940 |
| Wheat middlings | 7.500 | | | | |
| Dried buttermilk | 6.000 | 59.530 | 14.880 | | |
| Carbohydrate mixture* | 5.550 | 37.913 | 80.978 | 68.712 | 88.681 |
| N. A. fish meal | 5.500 | | | | |
| Alfalfa leaf meal | 2.500 | | | | |
| Ground limestone | 1.900 | 0.825 | 0.202 | 0.758 | 0.179 |
| Wheat bran | 1.500 | | | | |
| Sp. mineral mixture | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| $Ca_3(PO_4)_2$ | | 0.732 | 2.940 | 1.750 | 3.200 |
| Total | 100.000 | 100.000 | 100.000 | 100.000 | 100.000 |

* The carbohydrate mixture contained 50 per cent starch, 20 per cent dextrin, 20 per cent purified corn sugar, and 10 per cent sucrose.

TABLE 2

*Chemical analyses of the diets*

| | WATER | CRUDE PROTEIN (N × 6.25) | CRUDE ASH | ETHER EXTRACT | CRUDE FIBER | N-FREE EXTRACT |
|---|---|---|---|---|---|---|
| | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| Diet 1 | 4.94 | 19.69 | 6.47 | 3.72 | 3.11 | 62.07 |
| Diet 2 | 6.73 | 20.21 | 9.29 | 1.77 | 0.50 | 61.50 |
| Diet 3 | 4.23 | 5.45 | 5.55 | 0.28 | 0.19 | 84.30 |
| *Diet 4* | *4.01* | *22.23* | *5.47* | *2.43* | *1.01* | *64.85* |
| Diet 5 | 4.43 | 5.80 | 4.91 | 0.39 | 0.33 | 84.14 |

diets, respectively, with all of the protein from desiccated meat meal. The calcium and phosphorus content was adjusted so that each diet contained approximately 3.0 per cent of calcium and 1.2 per cent of phosphorus. In addition to these diets, during the feeding periods each bird was given approximately 5 cc. of cod-liver oil each day.

The high-protein diets were fed at levels such that the daily intake of nitrogen was 1.92 gram per bird, and the two low-protein diets were fed so that the daily intake was 0.48 gram of nitrogen per bird.

Each bird was placed in an individual wire cage 26 inches high, 21 inches long, and 17 inches wide. In some of the trials the excrement was collected on a hard rubber tray placed under the screen floor of the cage. In other trials the excrement was collected in a rubber bag attached to an

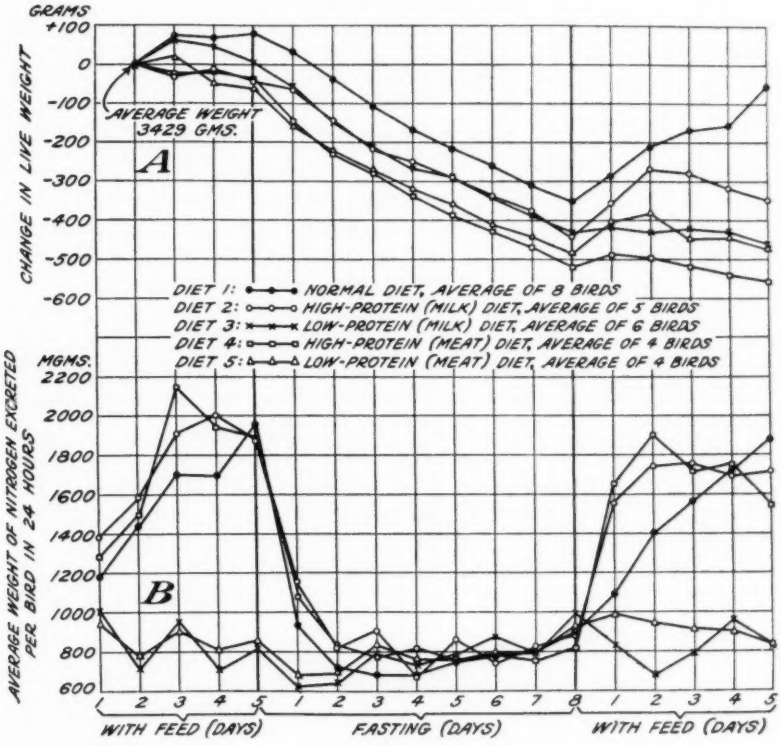


Fig. 1

aluminum ring, which in turn was fastened over the bird's vent by web straps passed under the wings and then across the shoulders where the straps were buckled together. Another strap attached to the ring was fastened around the base of the tail to prevent the collection bag from sliding down if the bird picked at the bag or the harness.

In all cases the birds were subjected to a preliminary feeding period of five days. This was followed by a fast of eight days. Following the fast

the diet used in the preliminary period was fed again for five days, to follow the course of nitrogen excretion after the fast. The birds were force-fed with the aid of a metal funnel and plunger, to assure a daily intake of the required quantity of feed. Water was given *ad libitum* throughout the trial.

Excrement was collected daily throughout the entire period of eighteen days. Nitrogen was determined on aliquot samples by the Kjeldahl method.

EXPERIMENTAL DATA. The data obtained in the several trials are summarized in figures 1A and 1B. The changes in live weight are shown by the curves in figure 1A, and the daily nitrogen excretion is shown by the curves in figure 1B.

DISCUSSION. It is evident that on the last day of the preliminary feeding period the birds on the three higher levels of protein intake were in approximate nitrogen equilibrium. The birds on the low-protein diets showed a negative balance of about 350 mgm. of nitrogen per day. It is interesting to note that at this lower level of protein intake the nitrogen excretion showed only a slight temporary change when feed was withdrawn.

The rates at which the birds in the different groups lost weight after feed was withdrawn were remarkably similar. The average total loss in weight during the fast, in each group, was just over ten per cent of the initial live weight of the birds. The type of feed fed during the preliminary period had virtually no effect on the loss of weight during the fast.

The data indicated that regardless of the level of protein intake, there was a drop in the quantity of nitrogen excreted after the feed was withdrawn. After this drop on the first day of fasting, the nitrogen excretion of birds previously fed on diets high in protein continued to decrease for one or two days, whereas that of the birds previously fed low-protein diets increased, until, on about the third day of fasting, the influence of previous feeding had disappeared.

From the third to the seventh days of fasting, inclusive, the level of nitrogen excretion was fairly constant. On the eighth day there seemed to be an increase in the quantity of nitrogen excreted. However, the differences in nitrogen excretion between individual birds became quite marked on the eighth day and the slight apparent increase probably had no significance.

The lag in nitrogen excretion on the first and second days of fasting on the part of the birds which had previously received the high-protein diets is undoubtedly caused by the presence of "deposit protein" (Lusk, 1923). The fact that these birds reached a fairly constant level of nitrogen excretion on the third day indicated that birds do not require such a long continued pre-experimental period to free the body of stored nitrogen as has been found necessary in the case of mammals by Hindhede (1926) and by Boas-Fixsen (1935).

The birds did not show a peak in nitrogen excretion on the second to fourth day of fasting, as has been reported for man. The nitrogen excretion curves are in general agreement with those reported for the domestic fowl by Voit (1901) and by Phillips, Ashworth and Brody (1932).

In order to learn whether or not there was any relation between the weight of nitrogen excreted and the weight of the experimental birds, the nitrogen excretion per unit of body weight was calculated for each bird and the resulting figures compared. No relationship was found.

During the feeding period which followed the fast, the only birds which showed a consistent gain in weight were those that received the normal diet. There was a small net gain for the birds receiving the high-protein (milk) diet, but no appreciable net gains on the other three diets. The birds receiving the normal diet were the only ones that showed a marked retention of nitrogen during the final feeding period. There was a small retention by birds receiving the other two high-protein diets. The birds on the two low-protein diets did not show any marked change in their nitrogen excretion when feeding was resumed.

## SUMMARY AND CONCLUSIONS

Twenty-seven Rhode Island Red cocks were divided into five groups and fed the following diets: 1, normal diet, protein from plant and animal sources; 2, high-protein diet, all of the protein from dried buttermilk; 3, low-protein diet, all of the protein from dried buttermilk; 4, high-protein diet, all of the protein from desiccated meat meal; 5, low-protein diet, all of the protein from desiccated meat meal.

The birds were force-fed for five days at fixed levels of nitrogen intake and then subjected to an eight-day fast. This was followed by a five-day feeding period. The daily nitrogen excretion was measured during the three periods.

The nitrogen excretion of all groups fell on the first day of the fast. On the second and third days the nitrogen elimination of the birds previously fed at the higher level of nitrogen intake continued to decrease, while that of the birds which had received the low-protein diets showed an increase on the third day. The influence of previous feeding disappeared on the third day, and three days of fasting were sufficient to free the body of stored nitrogen. From the third to the eighth days of the fast the quantity of nitrogen excreted daily was practically constant. The nitrogen curves differed from those observed for man in that there was no peak during the first few days of fasting.

The source of protein fed during the preliminary feeding period had no influence on the quantity of nitrogen excreted during the fast. However, the birds receiving the normal diet showed the greatest nitrogen retention and the largest gain in weight during the final feeding period.

## REFERENCES

BENEDICT, F. G. Carnegie Inst. of Washington. Publication no. 77, 1907.
BENEDICT, F. G. AND E. G. RITZMAN. Ibid., no. 377, 1927.
BOAS-FIXSEN, M. A. Nutrition Abs. and Reviews **4**: 447, 1935.
HINDHEDE, M. Biochem. J. **20**: 330, 1926.
HOWE, P. E., H. A. MATTILL AND P. B. HAWK. J. Am. Chem. Soc. **33**: 568, 1911.
KUCKEIN, F. Ztschr. f. Biol. **18**: 17, 1882.
LUSK, G. The science of nutrition. 3rd. ed. W. B. Saunders Company, Philadelphia and London, 1923.
MORGULIS, S. Fasting and undernutrition. E. P. Dutton & Co., New York, 1923.
PASHUTIN, V. A course of general and experimental pathology. Vol. 2, part 1. Trans. from Russian by Michel Groosenberg, 1902.
PHILLIPS, V. W., U. S. ASHWORTH AND S. BRODY. Mo. Agric. Exper. Sta. Res. Bull. 179, 1932.
SHIMANSKI, H. Ztschr. f. physiol. Chemie **3**: 396, 1879.
VOIT, E. Ztschr. f. Biol. **41**: 167, 1901.

# ETHER SOLUBLE LIPOID PHOSPHORUS LECITHIN AND CEPHALIN DISTRIBUTION IN THE DEVELOPMENT OF THE CHICK[1]

OTTO E. KUGLER

*From the Department of Zoology, University of Illinois, Urbana*

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In the classical work by Plimmer and Scott (1) on phosphorus metabolism in the developing hen's egg it was found that inorganic phosphorus increased at the expense of ether soluble phosphorus. Riddle (2) noted a marked absorption of phospholipids from the yolk during the last half of the incubation period. In the developing chick embryo Cahn (3) discovered that lipoid phosphorus rose gradually until the fifteenth day, after which it rose rapidly. Sharpe (4) and Nakamura (5) estimated the total choline in the developing egg. Their results were widely divergent.

The object of this investigation is to present a quantitative study of ether soluble lipoid phosphorus, lecithin, and cephalin in both yolk and embryo during the development of the fertile hen's egg.

EXPERIMENTAL. Fertile White Leghorn eggs obtained from the University of Illinois Poultry Farm during the summer of 1934 were selected in regard to uniformity of stock and weight and incubated under standard conditions. The fractions analyzed were chosen in conformity with the tables of magnitude in embryonic growth appearing in Needham's Chemical Embryology (6) and the embryos were checked against the morphological pictures in the normal tables of Duval (7) and Keibel and Abraham (8).

Yolks with yolk sacs were separated from the embryo fractions which included the amniotic and chorionic membranes and their contents. The ground fractions were mixed with three times their weight of anhydrous sodium sulphate, dried with stirring over a steam bath, and pulverized. The pulverized material was placed in extraction thimbles and each charge was extracted with 500 cc. of a mixture of three parts of absolute alcohol to one part of dry ether for twenty-four hours in a Soxhlet apparatus. The residue was reëxtracted for an additional two hours with a fresh alcohol-ether mixture. The dried alcohol-ether extract was dissolved in 50 cc. of ether and shaken with a saturated sodium chloride solution in a separatory

[1] Contribution from the Zoological Laboratory of the University of Illinois, No. 477.

funnel. The salt solution was drawn off and washed three times with 15 cc. of dry ether each time. The ether solutions were combined, the ether distilled off, and the residue dried to a constant weight in a vacuum desiccator.

Weighed amounts of the dried ether soluble extract were dissolved in a solution of three parts of absolute alcohol to one part of dry ether (in the

TABLE 1

| AGE | NUMBER OF SPECIMENS | AVERAGE PER SPECIMEN | | | | ETHER SOLUBLE LIPOID P per 100 GRAMS DRY WEIGHT | AVERAGE PER SPECIMEN | |
|---|---|---|---|---|---|---|---|---|
| | | Wet weight | Dry weight | Dried ether soluble extract | Ether soluble lipoid P | | Lecithin | Cephalin |
| **Yolks with yolk sacs** | | | | | | | | |
| *days* | | *grams* | *grams* | *grams* | *mgm.* | *mgm.* | *mgm.* | *mgm.* |
| 0 | 3 | 17.6 | 9.89 | 6.100 | 64.6 | 653.2 | 1089.8 | 362.3 |
| 3 | 3 | 21.7 | 9.98 | 6.156 | 65.8 | 660.3 | 1111.0 | 364.5 |
| 5 | 3 | 28.9 | 9.82 | 6.062 | 64.8 | 661.2 | 1064.0 | 361.0 |
| 7 | 6 | 26.5 | 9.54 | 5.874 | 62.2 | 652.0 | 1050.9 | 349.9 |
| 9 | 6 | 26.3 | 9.47 | 5.684 | 60.5 | 638.8 | 1021.4 | 336.5 |
| 11 | 6 | 21.4 | 8.99 | 5.401 | 57.5 | 639.6 | 969.1 | 319.5 |
| 13 | 9 | 19.1 | 8.21 | 4.930 | 52.2 | 635.8 | 879.4 | 291.6 |
| 15 | 6 | 15.7 | 7.22 | 4.268 | 45.6 | 631.6 | 769.1 | 254.0 |
| 17 | 7 | 14.8 | 6.81 | 3.210 | 34.1 | 500.7 | 577.6 | 191.4 |
| 19 | 4 | 11.7 | 5.50 | 2.502 | 26.8 | 487.3 | 452.2 | 149.2 |
| 20 | 4 | 10.6 | 5.20 | 2.374 | 25.1 | 482.7 | 425.3 | 140.7 |
| **Embryos with membranes and contents exclusive of yolk sacs** | | | | | | | | |
| 7 | 89 | 3.5 | 0.072 | 0.0248 | 0.25 | 347.2 | 4.2 | 1.4 |
| 9 | 53 | 6.3 | 0.125 | 0.0700 | 0.49 | 392.0 | 8.3 | 2.8 |
| 11 | 31 | 9.6 | 0.278 | 0.1032 | 1.01 | 363.3 | 17.0 | 5.5 |
| 13 | 31 | 15.3 | 0.726 | 0.2645 | 2.23 | 307.2 | 38.1 | 11.5 |
| 15 | 25 | 16.7 | 1.720 | 0.3784 | 3.73 | 216.9 | 63.5 | 20.6 |
| 17 | 9 | 22.7 | 3.264 | 0.8333 | 5.67 | 173.7 | 96.3 | 32.1 |
| 19 | 15 | 24.7 | 4.683 | 1.2905 | 6.84 | 146.1 | 116.1 | 36.3 |
| 20 | 8 | 25.2 | 5.543 | 1.5888 | 7.87 | 142.0 | 131.4 | 42.4 |

proportion of one gram of the extract to 100 cc. of solution). Aliquots of this mixture were evaporated to dryness in Pyrex test tubes and analyzed for ether soluble lipoid phosphorus according to the method of Fiske and Subbarrow (9).

The dried ether soluble extract was analyzed for lecithin and cephalin by the method of Brauns and MacLaughlin (10).

RESULTS AND DISCUSSION. The experimental results are to be found in table 1.

The yolk of the avian egg serves as the storehouse of phospholipids drawn upon by the developing embryo. As development proceeds there is a marked influx of water into the yolk from the albumen. This inflow reaches its highest level about the fifth day of incubation. By the nineteenth day the yolk sac is being incorporated into the body by an investing epithelium and in order to make a good separation it is necessary to cut the yolk sac from the mid-gut of the embryo. Murray (11) determined that embryos of seven days' incubation have a water content of 95 per cent while at nineteen days the water content is around 82 per cent. The amniotic fluid reaches its maximum content of about 2 cc. on the tenth day and only begins to fall about the twentieth day. The allantoic fluid rises regularly

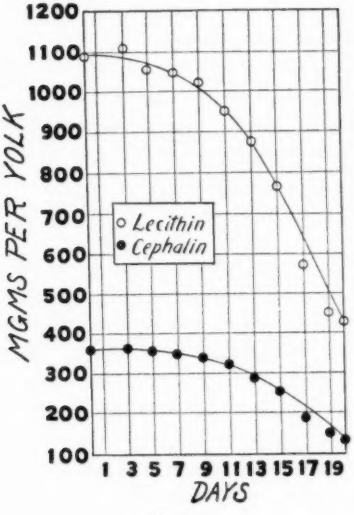


Fig. 1

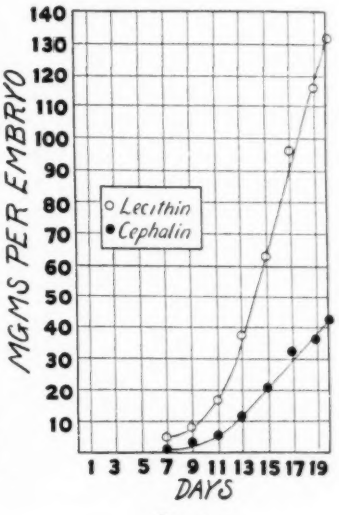


Fig. 2

attaining a maximum of about 6 cc. on the thirteenth day. From this point on its volume decreases slightly until the final desiccation of the egg before hatching.

In the yolk fraction ether soluble lipoid phosphorus drops from 65 mgm. at the beginning of the incubation period to 25 mgm. on the twentieth day. In absolute amount as well as in milligrams per 100 grams of yolk dry weight the fall is most rapid during the last week of incubation. This agrees with Riddle's observation on the utilization of phospholipids during the last half of the incubation period.

Ether soluble phosphorus in the embryo fraction rises from 0.25 mgm. on the seventh day to 7.87 mgm. on the twentieth day with a notice-

able inflection during the last week of incubation. This is in harmony with the findings of Cahn. In milligrams per 100 grams of embryo dry weight the lipoid phosphorus shows first a rise to the ninth day followed by a drop to the end of the incubation period. The early rise reflects the preponderance of development of the nervous system with its high lipoid content during the first nine days of chick embryogeny.

Lecithin and cephalin parallel each other (figs. 1 and 2) and show the same fluctuations as does ether soluble lipoid phosphorus during the developmental period. They maintain a ratio of approximately three to one respectively in both yolk and embryo fraction throughout the incubation period studied. Around 87 per cent of the ether soluble lipoid phosphorus was recoverable as lecithin and cephalin. Failure to account for all the lipoid phosphorus may be ascribed to losses inherent in the microanalytical procedures employed. Another probability is that some of the choline and ethanolamine may be split off the lecithin and cephalin molecules before the phosphorus, so that the latter would remain ether soluble.

From the lecithin results it is possible to calculate the combined choline. In the yolks it starts at 164 mgm. in the unincubated egg and falls in a manner like lipoid phosphorus to 65 mgm. on the nineteenth day. The embryo fraction contains 1.24 mgm. of combined choline on the ninth day and 19.8 mgm. on the nineteenth day.

Nakamura found that the total choline of the yolk rose from 79 mgm. in the unincubated egg to 94 mgm. in a nine day yolk then dropped to 22.5 mgm. in a nineteen day yolk. In the entire egg he found that the highest concentration of free choline was 3 mgm. at the time of hatching. In the embryos Nakamura found 0.35 mgm. choline on the ninth day and 7.02 mgm. on the nineteenth day. He was unable to find choline in the amniotic and allantoic fluids.

Sharpe used a colorimetric method for choline while Nakamura used a gravimetric one. Sharpe determined that the whole egg contained around 200 mgm. of total choline before incubation and 110 mgm. at the end of the incubation period. He found the maximum concentration of free choline to be around 15 mgm. for the whole egg. Sharpe's results tend to be in better agreement with the lecithin results calculated as choline in the present investigation than Nakamura's figures.

Inspection of the data in table 1 shows that phospholipid metabolism reaches its highest level during the time between the fifteenth and seventeenth days of incubation. This is the period of greatest yolk decrement and greatest embryo increment of lipoid phosphorus. It is also the period of greatest transformation of lipoid phosphorus into other forms and it coincides with the time of most active ossification of the embryonic bones. Transformation into the inorganic form is not the only fate of lipoid phosphorus. It is being incorporated into the body of the embryo as develop-

ment proceeds where it serves as an essential constituent particularly of cell membranes. Bloor has emphasized the function of phospholipids in the transport of fatty acids. Jost and Sorg (12) working on the whole egg were able to demonstrate a parallelism between the fall of phospholipid phosphoric acid and total fatty acids in the incubating hen's egg. They concluded that during fat metabolism the phospholipids are broken down but after the oxidation and splitting off of their acids are in part again supplied with such by the neutral fats.

## SUMMARY

1. In the embryonic development of the chick phospholipid metabolism reaches its highest stage between the fifteenth and seventeenth days of incubation.

2. Lecithin and cephalin parallel each other and show the same fluctuations as does ether soluble lipoid phosphorus during the developmental period of the chick. They maintain a ratio of approximately three to one respectively in the yolk as well as in the embryo fraction throughout the incubation period studied.

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## REFERENCES

(1) Plimmer and Scott. J. Physiol. **38:** 247, 1909.
(2) Riddle, O. This Journal **41:** 409, 1916.
(3) Cahn, T. Inaug. Diss. Paris, 1928.
(4) Sharpe, J. S. Biochem. J. **18:** 151 (1924).
(5) Nakamura, Y. Ztschr. f. physiol. Chem. **177:** 34, 1928.
(6) Needham, J. Chemical embryology. Cambridge, 1931.
(7) Duval, M. Atlas d'Embryologie. Paris, 1889.
(8) Keibel and Abraham. Normaltafeln zur Entwicklungsgesch. d. Hühnes. Jena, 1900.
(9) Fiske and Subbarrow. J. Biol. Chem. **66:** 375, 1925.
(10) Brauns and MacLaughlin. J. Am. Chem. Soc. **42:** 2339, 1920.
(11) Murray, H. A. J. Gen. Physiol. **9:** 39, 1926.
(12) Jost and Sorg. Pflüger's Arch. **231:** 143, 1932.

# THE POSITION OF THE OXYGEN DISSOCIATION CURVE OF HUMAN BLOOD AT HIGH ALTITUDE[1]

ANCEL KEYS, F. G. HALL AND E. S. GUZMAN BARRON

*From the Fatigue Laboratory, Harvard University, the Department of Zoology, Duke University, and the Lasker Foundation for Medical Research, and the Department of Medicine, University of Chicago*

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The relative constancy of the fundamental affinity between hemoglobin and oxygen at sea level has been the cornerstone of the theoretical exploration of the respiratory cycle. At a given hydrogen-ion concentration the variations in the oxygen dissociation curve between individual men are not much beyond the limits of experimental error, but differences in pH, such as can be produced by changed breathing conditions, alter the position of the curve in an orderly manner to an extent which may be physiologically significant.

At high altitude, other things being equal, the increased pulmonary ventilation would be expected to make the blood abnormally alkaline so that the dissociation curve *in vivo* would tend to shift to the left, i.e., there would be an increased affinity of the hemoglobin for oxygen. A changed position of the oxygen dissociation curve as a possible major factor in acclimatization to high altitude was first seriously suggested by Barcroft and co-workers (1922). Previously Douglas, Haldane, Henderson and Schneider (1913) reported no marked change at 4,300 meters in the position of the $O_2$ curve of blood equilibrated with alveolar air. The results of Barcroft's Anglo-American Expedition to Peru (op. cit.), however, tended to show that after living some weeks at 4,300 meters there is a shift of the curve to the left as compared to sea level. Part of this shift was

[1] Paper no. 3 from the International High Altitude Expedition to Chile. The Expedition was supported by grants from the Fatigue Laboratory and the Milton Fund, Harvard; Duke University; Copenhagen University; King's College, Cambridge University; Columbia University; the National Research Council, the Royal Society, London; the Corn Industries Research Foundation; the Rask-Ørsted Fund, Copenhagen; the Josiah Macy Foundation and the American Association for the Advancement of Science. We are also much indebted to the Chile Exploration Company and to its officers in New York and in Chile, to Señores Hipolito and Juan Carrasco of the S. I. A. M. Carrasco, to Messrs. Packard and Bell of the Poderosa Mining Company, to the Ferro-Carril de Antofagasta a Bolivia, and to the many people, officials and private citizens, who facilitated our work in Chile.

explained as a result of increased alkalinity of the blood, but, in addition, it was suggested that there may be a real change in the affinity of Hb for $O_2$ which is independent of pH (see Barcroft, 1925).

More recently, Dill and co-workers (1931) made some investigations at Leadville on blood from acclimatized subjects at 3,280 meters and from one subject after 4 days at 4,200 meters. Their results offered no support for Barcroft's suggestion; the position of the dissociation curve, within the limits of error, was unchanged, or, if anything, tended to show a possible slight *decrease* in the affinity of Hb for $O_2$. In addition they studied electrolyte distribution between cells and plasma and found no abnormalities which would jeopardize the validity of their conclusions. However, it could be objected that perhaps the altitude was too low to produce effects similar to those discussed by Barcroft. On the other hand, Buikov and Martinson (1933) reported a general corroboration of Barcroft at the very moderate altitudes of 600 to 2,100 meters; the adequacy of their data and technique is open to question.

It is well known that many animal species have hemoglobins which differ in their oxygen-combining properties and, in some cases at least, these differences are correlated with differing respiratory requirements (cf., e.g., Macela and Seliškar, 1925; Dill, Edwards, Florkin and Campbell, 1932). Recently the general idea of the existence, within a single species of animal or even in a given individual, of various forms of hemoglobin with differing affinities for oxygen has gained some currency through the work of Geiger (1931) and of Brinkman (1933; Brinkman and Jonxis, 1935) on the rate of alkali denaturation of human hemoglobin and especially through the work of Barcroft's laboratory on fetal blood (McCarthy, 1933; Hall, 1934b, c; Barcroft and co-workers, 1934). The latter work demonstrated a marked superiority in oxygen affinity of the fetal blood of the goat and the blood of the incubating chick as compared to the adult forms of those animals. The adaptive nature of this peculiarity of the blood of embryos seems probable in view of the oxygen paucity of their environments. The intriguing possibility of a similar adaptation of the blood of man at high altitude has been argued by Barcroft, notably in an address with the apt title, "Everest in Utero."

The International High Altitude Expedition to Chile in 1935 included in its program a more thorough investigation of the affinity of hemoglobin for oxygen as seen from dissociation curves. The present paper presents results obtained by direct tonometric methods on the blood from the 10 members of the Expedition at altitudes up to 6,140 meters and from 11 permanent residents at 3,660 and 5,340 meters. Dissociation curves obtained by the spectro-comparator method of Hall (1934 a) on laked blood solutions are presented in a separate paper in this series (Hall, 1936).

PROCEDURE AND METHODS. At sea level, before leaving for the field,

complete oxygen dissociation curves at pH of the cells ($pH_c$) = 7.10 and partial curves at $pH_c$ = 6.83 and 7.40 were obtained for 7 members of the Expedition. Similar curves were obtained for 2 other members (Barron and McFarland) about 15 weeks after their return to sea level. On all these men oxygen saturation and $pH_c$ were determined on the arterial blood as drawn in basal rest. In the case of Matthews there was no opportunity to obtain strictly comparable data at sea level.

The itinerary and general description of the stay of the Expedition at high altitude are given elsewhere (Keys, 1936). During a period of 14 weeks 5 stations were occupied (table 1).

At each of the stations the following data, in general, were secured for arterial blood drawn in basal rest from the various members: (1) $O_2$ capacity, (2) $O_2$ and $CO_2$ contents, (3) two points on the oxygen dissociation curve at body temperature and at $CO_2$ tensions close to the alveolar

TABLE 1

| STATION | PERIOD OF OCCUPANCY | CORRECTED BAROMETRIC PRESSURE | ALTITUDE | |
|---|---|---|---|---|
| | | | Meters | Feet |
| Chuquicamata | April 8 to June 4 | 543 | 2,810 | 9,200 |
| Ollagüe | June 5 to June 13 and June 25 to July 18 | 489 | 3,660 | 12,020 |
| Collahuasi (Montt) | June 13 to June 25 | 429 | 4,700 | 15,440 |
| Aucanquilcha | June 26 to July 15 | 401 | 5,340 | 17,500 |
| Punta de Cerro | June 29 to July 14 | 356 | 6,140 | 20,140 |

tension, (4) plasma $CO_2$ content, (5) check pH values on some but not all samples, (6) cell volume by hematocrit, (7) plasma protein concentration by refractometer, (8) data on electrolyte distribution. Analyses and equilibrations were made on the day of collection, generally within at most a few hours, the blood meanwhile being stored under oil at 0°C.

In addition to the studies on the Expedition members in rest, similar studies were made in 4 instances on arterial blood drawn in work and in 11 instances on arterial blood drawn in basal rest from residents of the high regions.

Dr. D. B. Dill has summarized, with comments, in the footnote below[2]

[2] The procedure used to determine the position of the oxygen dissociation curve was as follows:

(a) Draw arterial blood and stir it gently with heparin under oil.

(b) Determine on the Van Slyke apparatus its $CO_2$ and $O_2$ contents and its oxygen capacity.

(c) Determine by equilibration with suitable gas mixtures at body temperature

the procedure and calculations used to determine the position of the oxygen dissociation curve.

The essential relations and the steps used in the calculations are given in figures 1, 2 and 3A; the application to a typical case is given in figures 3B and 4. The $pH_e$ correction was justified by the fact that both the results of the present Expedition and those of the Leadville Expedition (Dill

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points on the oxygen dissociation curve near the $pCO_2$ of arterial blood with one point above and one point below its $pO_2$.

(d) Determine $CO_2$ in the equilibrated blood samples and proceed to derive the $CO_2$ dissociation curve of arterial blood, making use of figure 1 and the data of Henderson et al. (1931).

(e) Using the $CO_2$ curve thus derived, apply the arterial $CO_2$ content and determine the arterial $pCO_2$.

(f) Using figures 2 and 3B, obtain the $pH_e$ values for equilibrated blood samples and arterial blood.

(g) From the determined effect of pH on the affinity of the blood for oxygen, as in figure 3B, correct the oxygen dissociation curve to the arterial $pH_e$. The position of the curve at the standard $pH_e$ value, 7.10, may also be estimated.

The following comments may be made on these steps:

(a) Looney and Childs (1934) have pointed out the danger of gas exchange through oil and favor the storage of blood in syringes. We found, however, that when the volume of blood is large (usually at least 25 cc. was transferred under oil into a 50 cc. centrifuge tube) the changes in $CO_2$ and $O_2$ content are negligible.

(b) Some difficulties were experienced at the first station (Chuquicamata) in getting satisfactory results with the combined $CO_2$ and $O_2$ method of Van Slyke. In all later analyses separate determinations of $CO_2$ and $O_2$ were made in duplicate on 0.5 cc. samples.

(c) The usual method of equilibration with Barcroft tonometers of about 300 cc. capacity was used, the gas mixture being analysed on the Haldane machine after equilibration (Henderson, 1928, Appendix). When equilibrium was reached the tonometer was turned to a vertical position and, while submerged, was covered with a towel. After withdrawal from the bath a second (dry) towel was wrapped around the whole, and connection was made with a 5 cc. oiled syringe containing 1 cc. of mercury. Some mercury was pushed through the sidearm, the cock turned and all the blood drawn into the syringe. The small-bore rubber connection was left on the syringe and closed with a glass plug as soon as the tonometer was disconnected. When a sample was to be taken from the syringe the mercury was used for mixing, the syringe was turned upright, the plug removed and blood pushed directly into the pipette. This method was easily carried out under primitive conditions and was economical of time, mercury and glassware.

(d) Figure 1 is based on empirical observations, but it is in close accord with the data of Henderson et al. (1931).

(e) Figure 3A is based on the solubility coefficients of $CO_2$ in serum and cells established by Van Slyke, Hastings, Sendroy and Neill (1928). Figure 2 is based on the paper of Henderson and associates (1931) and a corresponding set of tables for reduced blood not yet published. In its construction it was assumed that $pK_e$ varies with oxygenation as found by Stadie and Hawes (1928).

and co-workers, 1931) indicate that the effect of pH on the oxygenation of hemoglobin is not affected by altitude. Determinations on the blood of all the members of the present Expedition gave a mean $\Delta$ log. $pO_2$ per 0.1 $pH_c$ of 0.0568 with a range from 0.055 to 0.059.

Figure 1 gives the effect of oxygenation on the $CO_2$ dissociation curve at $pCO_2 = 40$ mm. Figure 3A gives the carbonic acid $CO_2$ content per mm. of $CO_2$ pressure from the oxygen capacity. Figure 3B gives the effect

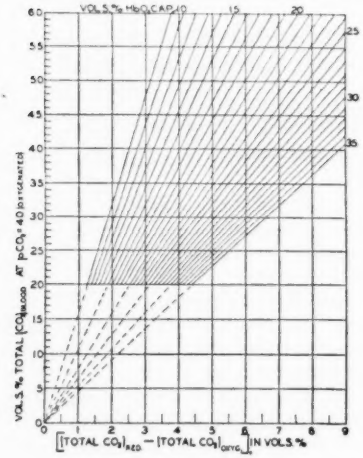


Fig. 1

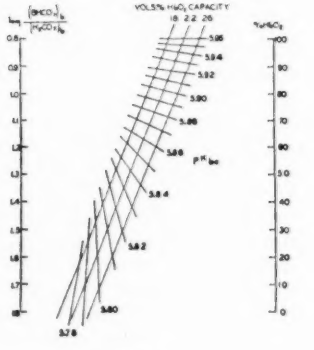


Fig. 2

Fig. 1. Chart used for locating the position of the $CO_2$ dissociation curve. From empirical data from the Fatigue Laboratory. The relation is somewhat uncertain below 20 vols. per cent total $CO_2$.

Fig. 2. Alignment chart used for the calculation of $pH_c$ from per cent $HbO_2$, $O_2$ capacity and the ratio of total bicarbonate of the blood to the dissolved carbonic acid of the blood. A straight line connecting the appropriate points on the ordinate for per cent $HbO_2$ and $\log \frac{(BHCO_3)_b}{(H_2CO_3)_b}$ indicates $pK_c$ at the intersection with the proper $O_2$ capacity. By Dill from data from the Fatigue Laboratory.

of $pH_c$ on the oxygen affinity of the blood of one subject (E. H. C.). From figure 2 the $pK_{bc}$ is determined and then $pH_c = pK_{bc} + \log \frac{(BHCO_3)_b}{(H_2CO_3)_b}$. In figure 4 are given, as an example of the method, the sea level points for the blood of E. H. C. at $pH_c = 7.36$, 7.10 and 6.84 (from which fig. 3B was constructed), together with the oxygen dissociation points determined on the blood of E. H. C. at 5,340 meters; the $pH_c$ values are those found in the equilibrations. Finally, in figure 4, the dissociation points at 5,340

meters have been corrected to $pH_c = 7.10$ (by the use of fig. 3B) and the broken line is the oxygen dissociation curve at this standard $pH_c$.

RESULTS. The principal results are presented in table 2, in which are given $pO_2$ values required to produce half saturation both at the constant $pH_c = 7.10$ and at the $pH_c$ found for the arterial blood at the place. The values at arterial $pH_c$ are those for equilibration at approximately the true

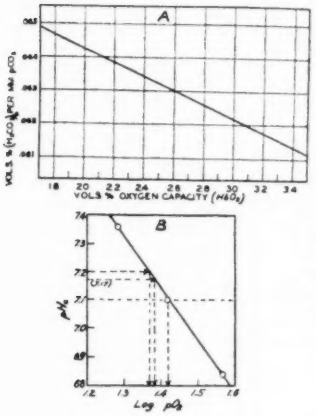


Fig. 3

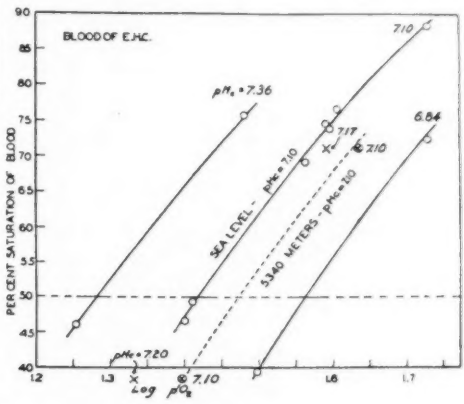


Fig. 4

Fig. 3. *A*. Relation of $(H_2CO_3)_b$ per millimeter $pCO_2$ to total hemoglobin of the blood as expressed in volumes per cent $O_2$ capacity. Data from Van Slyke et al., 1928.

*B*. Relation of $pO_2$ to $pH_c$ at $Hb = HbO_2$ as derived from the sea level results in *A*. The broken lines are the intercepts for finding the $pO_2$ correction for the transformations, at $Hb = HbO_2$, from $H_c = 7.20$ and $pH_c = 7.17$ to $pH_c = 7.10$.

Fig. 4. Correction of dissociation points to constant $pH_c$, and the construction of the partial dissociation curve at the high altitude stations. Blood of E. H. C. at Boston at 3 levels of $pH_c$. The results of two equilibrations of the blood of E. H. C. at 'Quilcha, 5,343 meters, are indicated by crosses with the $pH_c$ values as found. The crosses within circles are these points corrected to $pH_c = 7.10$ by means of the diagram C. The broken line is the partial dissociation curve for E. H. C. at 5,343 meters at $pH_c = 7.10$; it is the best approximation satisfying the requirements of fitting the two points and possessing a slope in agreement with the other dissociation curves.

rectal temperatures at the stations. At Boston the standard temperature of 37.0°C. was used for all except Barron and McFarland; in the latter cases the rectal temperature was used for equilibration. The rectal temperatures were always slightly less than 37.0°, ranging down to 36.5° and averaging about 36.8°. The values for $pO_2$ at $pH_c = 7.10$ given in the tables are corrected to 37.0° by the use of the data of Brown and Hill

(1923). This correction is small—for the difference between 36.0 and 37.0° the $pO_2$ is increased about 0.8 mm., so the correction amounts to +0.4 mm. in the extreme case with a mean correction of slightly less than +0.2 mm.

TABLE 2

*Position of the oxygen dissociation curve at various altitudes*

Values for $pO_2$ at half saturation are in mm. Hg and are at 37.0°C. for constant $pH_c = 7.10$ and at the observed rectal temperature for the $pH_c$ of the arterial blood as drawn.

| SUBJECT | Days after arrival | Arterial $pH_c$ | $HbO_2$ capacity vols. per cent | $pO_2$ for Hb. = $HbO_2$ | |
|---|---|---|---|---|---|
| | | | | arter. $pH_c$ | $pH_c$ = 7.10 |
| **AT SEA LEVEL (BOSTON)** | | | | | |
| Barron | 100 | 7.08 | 19.42 | 25.3 | 25.1 |
| Christensen | | 7.12 | 19.33 | 26.2 | 26.6 |
| Dill | | 7.10 | 18.22 | 26.8 | 26.9 |
| Edwards | | 7.10 | 19.62 | 25.5 | 25.7 |
| Forbes | | 7.15 | 20.37 | 25.3 | 26.1 |
| Hall | | 7.11 | 22.06 | 26.8 | 27.1 |
| Keys | | 7.11 | 18.60 | 26.9 | 27.2 |
| McFarland | 100 | 7.10 | 20.87 | 26.1 | 26.3 |
| Talbott | | 7.11 | 20.66 | 25.0 | 25.3 |
| Mean | | 7.11 | 19.91 | 25.98 | 26.28 |
| **AT 3.66 KM., B. P. = 489 MM. (OLLAGÜE)** | | | | | |
| Barron | 1 | 7.18 | 23.15 | 22.5 | 25.5 |
| Christensen | 6 | 7.22 | 24.92 | 26.0 | 30.9 |
| Dill | 7 | 7.16 | 20.88 | 27.8 | 30.1 |
| Edwards | 7 | 7.15 | 23.47 | 24.8 | 26.6 |
| Forbes | 2 | 7.17 | 21.68 | 23.6 | 26.4 |
| Hall | 7 | 7.22 | 24.65 | 24.1 | 28.2 |
| Keys | 3 | 7.13 | 21.06 | 26.6 | 28.0 |
| Talbott | 1 | 7.14 | 22.52 | 26.3 | 27.6 |
| Mean | 4.3 | 7.17 | 22.69 | 25.30 | 27.91 |
| *In work* | | | | | |
| Matthews | 2 | 7.08 | 24.78 | 28.9 | 28.4 |
| Talbott | 3 | 7.05 | 25.55 | 30.8 | 28.0 |
| Mean | 2.5 | 7.07 | 25.17 | 29.85 | 28.20 |
| *Residents* | | | | | |
| Carrasco | | 7.12 | 24.17 | 25.5 | 26.4 |
| Aramayo* | | 7.07 | 25.83 | 31.5 | 30.5 |
| Tomasa* | | 7.08 | 25.00 | 27.3 | 27.0 |
| Mean | | 7.09 | 25.00 | 28.10 | 27.97 |

| SUBJECT | Days after arrival | Arterial $pH_c$ | $HbO_2$ capacity vols. per cent | $pO_2$ for Hb. = $HbO_2$ | |
|---|---|---|---|---|---|
| | | | | arter. $pH_c$ | $pH_c$ = 7.10 |
| **AT 2.81 KM., B. P. = 543 MM. (CHUQUICAMATA)** | | | | | |
| Barron | 13 | 7.12 | 22.64 | 24.5 | 25.3 |
| Christensen | 53 | 7.16 | 21.40 | 25.1 | 27.5 |
| Dill | 14 | 7.14 | 19.93 | 23.7 | 25.3 |
| Edwards | 54 | 7.16 | 22.75 | 22.6 | 24.6 |
| Forbes | 56 | 7.14 | 22.80 | 23.7 | 25.2 |
| Hall | | | | | |
| Keys | 51 | 7.09 | 19.64 | 26.6 | 26.4 |
| McFarland | 10 | 7.14 | 21.67 | 24.3 | 25.9 |
| Talbott | 14 | 7.12 | 22.19 | 26.3 | 27.2 |
| Mean | 33.1 | 7.13 | 21.63 | 24.60 | 25.93 |
| **AT 4.71 KM., B. P. = 429 MM. (COLLAHUASI)** | | | | | |
| Barron | 1 | 7.16 | 24.80 | 25.8 | 28.3 |
| Barron | 8 | 7.13 | 25.56 | 26.9 | 28.2 |
| Christensen | 4 | 7.23 | 24.31 | 26.0 | 30.9 |
| Dill | 9 | 7.15 | 22.10 | 27.9 | 30.1 |
| Edwards | 6 | 7.14 | 24.14 | 25.0 | 26.6 |
| Forbes | 5 | 7.21 | 23.68 | 25.1 | 29.2 |
| Hall | 11 | 7.16 | 25.86 | 26.0 | 28.2 |
| Keys | 7 | 7.12 | 23.14 | 24.1 | 25.1 |
| McFarland | 2 hr. | 7.16 | 22.46 | 23.7 | 26.0 |
| McFarland | 9 | 7.17 | 25.40 | 25.8 | 28.3 |
| Matthews | 4 | 7.19 | 24.37 | 27.6 | 31.3 |
| Talbott | 1 | 7.13 | 23.32 | 28.1 | 29.6 |
| Talbott | 7 | 7.12 | 23.80 | 28.3 | 29.7 |
| Mean | 5.5 | 7.16 | 23.91 | 26.18 | 28.81 |
| *In work* | | | | | |
| Matthews | 7 | 7.14 | 26.30 | 28.5 | 29.9 |
| Talbott | 6 | 7.15 | 24.91 | 28.0 | 30.0 |
| Mean | 6.5 | 7.15 | 25.61 | 28.25 | 29.95 |

TABLE 2—*Concluded*

| SUBJECT | AT 5.34 KM., B. P. = 401 MM. ('QUILCHA) | | | | | SUBJECT | AT 6.14 KM., B. P. = 356 MM. (PUNTA) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Days after arrival | Arterial $pH_c$ | $HbO_2$ capacity, vols. per cent | $pO_2$ for Hb. = $HbO_2$ | | | Days after arrival | Arterial $pH_c$ | $HbO_2$ capacity vols. per cent | $pO_2$ for Hb = $HbO_2$ | |
| | | | | arter. $pH_c$ | $pH_c$ = 7.10 | | | | | arter. $pH_c$ | $pH_c$ = 7.10 |
| Barron | 5 | 7.18 | 25.40 | 26.7 | 29.9 | Barron† | 2 hr. | 7.04 | 27.12 | 30.1 | 28.1 |
| Christensen | 4 | 7.17 | 23.70 | 26.9 | 29.7 | Barron | 1 | 7.16 | 26.61 | 25.8 | 28.1 |
| Dill | 4 | 7.12 | 22.94 | 29.1 | 30.1 | Dill† | 2 hr. | | 22.59 | | 30.8 |
| Edwards | 16 | 7.13 | 25.93 | 25.0 | 26.4 | Edwards† | 2 hr. | 7.07 | 25.37 | 28.5 | 27.6 |
| Forbes | 5 | 7.13 | 23.73 | 27.1 | 28.4 | Forbes | 1 | 7.16 | 23.66 | 27.4 | 29.8 |
| Hall | 1 | 7.14 | 25.55 | 28.3 | 30.2 | Forbes§ | 2 | 7.16 | 24.66 | 27.5 | 30.1 |
| Keys | 3 | 7.17 | 23.72 | 26.6 | 29.5 | Hall† | 2 hr. | 7.21 | 25.51 | 24.3 | 28.3 |
| McFarland | 3 | 7.14 | 24.67 | 28.1 | 29.8 | Keys | 3 | | 24.38 | | 30.5 |
| Matthews | 3 | 7.12 | 23.70 | 25.8 | 27.0 | Keys | 6 | 7.12 | 23.25 | 26.9 | 27.9 |
| Talbott | 3 | 7.09 | 24.95 | 28.9 | 28.9 | McFarland | 2 | 7.18 | 26.33 | 27.5 | 30.7 |
| Mean | | 7.14 | 24.43 | 27.3 | 28.99 | Matthews | 3 | 7.12 | 24.69 | 27.7 | 28.8 |
| Alcaino | | 7.15 | 29.77 | 25.5 | 27.4 | Matthews | 6 | 7.11 | 24.42 | 28.1 | 28.8 |
| Alcio | | 7.06 | 29.25 | 28.5 | 27.3 | Talbott‡ | 1 | 7.07 | 25.27 | 30.1 | 29.2 |
| Bastias | | 7.08 | 30.70 | 24.4 | 24.0 | Mean | | 7.13 | 24.90 | 27.6 | 29.13 |
| Campos | | 7.07 | 32.72 | 27.5 | 26.6 | | | | | | |
| Fritz | | 7.11 | 24.62 | 27.2 | 27.6 | | | | | | |
| Heredia | | 7.06 | 34.20 | 29.0 | 27.8 | | | | | | |
| Martinez | | 7.13 | 19.87 | 25.8 | 27.1 | | | | | | |
| Troncoso | | | 32.54 | | 27.7 | | | | | | |
| Mean | | 7.09 | 29.21 | 26.8 | 26.94 | | | | | | |

* Chronic mountain sickness. Aramayo was from 4.71 km. visiting at 3.66 km.

† After climbing from 5.43 km.

‡ Ill of acute mountain sickness.

§ A period of 2 days at 5.43 km. intervened before Forbes encamped at 6.14 km. for the second stay.

The calculated values for $pH_c$ may be questioned. Rubowitz (1933) claims that the isoelectric point of Hb (and also $HbO_2$) is changed at high altitude; it could be argued that the chemical relationships at sea level, on which the calculation is based, may not hold at high altitude. In answer, comparison may be made between the observed and calculated values for the whole blood ($pH_s$). The general question of the reaction of the blood and the observed and calculated values for $pH_s$ at high altitudes are presented elsewhere (Forbes, Keys and Hall, 1936). In 26 experiments above 12,000 feet, where both methods were used, the mean value for $pH_s$ observed was 7.449 and the mean calculated $pH_s$ was 7.428. The glass electrode assembly used in the field yielded values which were not always above suspicion as to absolute accuracy but, even assuming that the small differences between the observed and calculated values were

always due to fault of the calculation method, the effect on the dissociation curve is slight. It will be remembered that a difference in $pH_s$ of 0.1 corresponds to a smaller difference, about 0.08, in $pH_c$ (cf., e.g., Henderson, Dill, Edwards and Morgan, 1931). Moreover, the observed differences apparently were not related to altitude. These points are shown in table 3; the positions of the dissociation curves are moved to the right by an average of 0.6 mm. $pO_2$ at half saturation if the $pH_s$ observed is accepted and a correction made accordingly. In tables 2 to 8 the calculated values have been used throughout.

GENERAL DISCUSSION. At none of the stations was there any increase in the affinity, at constant $pH_c$, of hemoglobin for oxygen in our party. Moreover, the values obtained for the permanent residents are certainly not to the left of the normal values for sea level. It can be stated with

TABLE 3

*Differences between observed and calculated $pH_s$, together with the effect on $pH_c$ and on $pO_2$ at half saturation, assuming observed $pH_s$ to be correct*

| STATION | NUMBER OF EXPERIMENTS | $pH_s$ OBSERVED—$pH_s$ CALCULATED | CORRECTION, $pH_c$ CALCULATED | MEAN $pO_2$ AT Hb. = $HbO_2$ | | | |
|---|---|---|---|---|---|---|---|
| | | | | At arterial $pH_c$ | | At $pH_c$ = 7.10 | |
| | | | | Original | Corrected | Original | Corrected |
| Ollagüe | 3 | +0.033 | +0.026 | 25.2 | 26.1 | 27.9 | 28.8 |
| Collahuasi | 9 | +0.010 | +0.008 | 26.2 | 26.5 | 28.6 | 28.9 |
| 'Quilcha | 8 | +0.021 | +0.017 | 27.3 | 27.9 | 29.0 | 29.6 |
| Punta | 6 | +0.030 | +0.024 | 27.6 | 28.4 | 29.1 | 29.9 |
| Mean | | +0.021 | +0.017 | 26.6 | 27.2 | 28.7 | 29.3 |

confidence, then, that at high altitude the fundamental $O_2$ dissociation curve does not shift to the left. On the contrary, there is a uniform tendency for the curve to shift increasingly to the right of the sea level position. In figure 5B are plotted the changes with altitude in the $pO_2$ required to produce half saturation at $pH_c$ = 7.10 and 37.0°C., and to these data are added the results of the Leadville Expedition—the only comparable data from other work. Allowing for the delicacy of the measurements, the picture is surprisingly consistent.

Speculation as to the cause of this apparent diminution in the hemoglobin affinity for oxygen is not very profitable. It can scarcely be attributed to methodical error in the method of calculation because the entire procedure was constant for all stations. The general agreement between observed and calculated pH has been mentioned above; if the high altitude curves should be "corrected" for the average discrepancy between observed and calculated pH they would be still further shifted to the right

—0.6 mm. in $pO_2$ on the average—and, since it has been found that the calculation method gives accurate results at sea level, the change of the midpoint of the dissociation curve at high altitude would be increased by 0.6 mm. in $pO_2$.

The dissociation curves of the laked blood in buffer solution (Hall, 1936) do not show this tendency but appear to be unchanged with altitude.

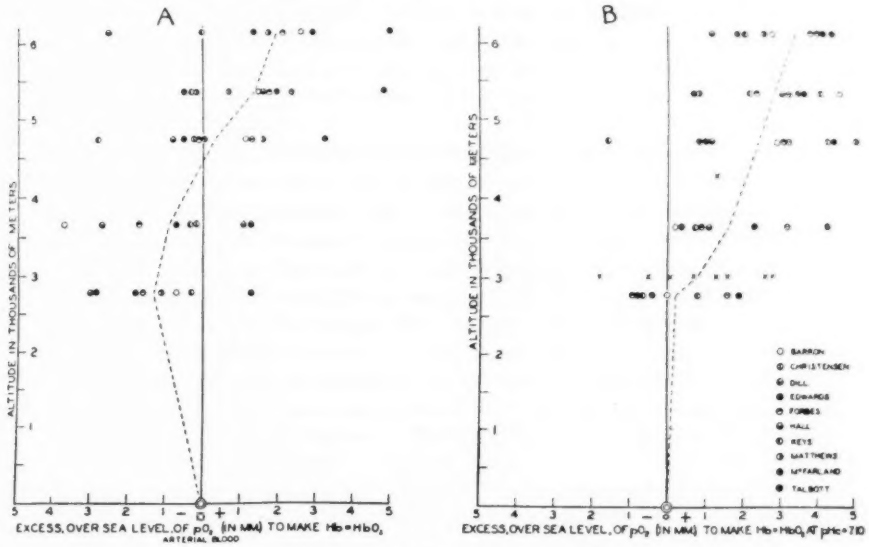


Fig. 5. Change, with altitude, of the position of the half saturation point of the oxygen dissociation curve at $pH_c = 7.10$ and temperature = 37.0°C., and at arterial $pH_c$ and rectal temperature. Circles, present results; crosses, data from the Leadville Expedition. Each point is the mean result for one individual. The sea level reference for Matthews was assumed to be the same as the mean of the sea level values for the other nine members of the Expedition. The broken line connects the mean values at each altitude. The values at the highest station for Dill, Edwards and Hall were not included in calculating the mean because these subjects only stopped at this altitude for an hour or two before the blood was drawn. In 5A the value for Talbott was not included in calculating the mean at 6.14 km. because he was ill of mountain sickness at the time.

The electrolyte distribution between cells and plasma is discussed elsewhere in this series (Talbott and Consolazio, 1936); it is enough to note here that the bicarbonate ratio, $r_{HCO_3}$, was within normal limits at all stations. Also, though the hemoglobin concentration of the blood was greatly increased, the concentration of Hb in the cells was little changed (Talbott, 1936).

Litarczek, Aubert and Cosmulesco (1933) claim that the affinity of Hb for $O_2$ can be altered, independently of pH, by the presence of substances like glutathione in the cells. However, even granting that this may be true and there may have been alterations with altitude in the concentrations of such substances, it is not clear why there should be any difference between the results with whole blood and with the laked blood solutions.

The relation between the "physiological" $O_2$ dissociation curves at high altitudes and those at sea level is somewhat different. The changes with altitude are shown in figure 5A, which shows that, in our party, the curves tended to shift to the left at our two lower stations and to shift progressively to the right of the sea level values as we moved up to the higher stations.

These results are not entirely inconsistent with those of Haldane or those of Barcroft. At the altitudes at which they worked, our results indicate a small shift to the left of the sea level and sufficient individual variation to give the possibility of obtaining results similar to either those of Barcroft or Haldane with small series and the older techniques.

It is of interest to note that the average line for our results crosses the line of no change at 4,500 meters. This may or may not be related to the general feeling common among mountain dwellers and mountain climbers, and in which we would concur in general so far as subjective experiences go, that somewhere around this altitude there is a transition zone above which the maintenance of a reasonably normal life becomes very much more difficult.

*Comparison with residents.* Comparison of our party with the permanent residents at Ollagüe is not very satisfactory because only one of the 3 resident subjects, Carrasco, could be considered normal. However, in any case, at $pH_c = 7.10$ there is no perceptible difference between the two groups and at arterial $pH_c$ the residents' dissociation curves are to the right of our average.

At 'Quilcha the comparison is much more satisfactory and it is clear that at constant $pH_c$ our hemoglobin had a smaller affinity for oxygen than did that of the residents. This difference must be considered as probably significant; only two men in our group of ten had curves which fell within the range of the miners' dissociation curves. Since the residents were undeniably better acclimatized than our group, it must be concluded that probably part of the shift to the right of our curves at constant $pH_c$ may be referred to incomplete acclimatization. The comparisons at 'Quilcha become more interesting when it is noted that though the mean $pH_c$ of the residents is very close to the normal value for sea level, our average was sufficiently more alkaline than at sea level so that our "physiological" dissociation curves—and our arterial $O_2$ saturations—approximated those of the residents.

Individual differences in dissociation curves do not, at present, fall into any orderly picture; their possible relation to degree of adaptation is discussed more at length elsewhere (Keys, Matthews and Forbes, 1936).

*Muscular exercise.* In work on the bicycle ergometer at an intensity close to the maximum that could be maintained by the subjects, there was no significant change in the $pO_2$ required to produce half saturation at $pH_c = 7.10$, either at Ollagüe or at Collahuasi. The arterial blood samples were taken during work near the end of a 10-minute period of exercise. At Ollagüe the $pH_c$ of the arterial blood of both subjects was almost 0.1 pH more acid than in rest and, accordingly, the $O_2$ dissociation curves are considerably to the right of the values for the arterial blood in rest but not more so than would be the case for similar exertion at sea level. At the higher altitude of Collahuasi, on the other hand, the $pH_c$ values are much the same as in rest—more alkaline than in rest at sea level—and the "physiological" dissociation curves are practically the same as in rest. This peculiarity would seem to be accounted for partly by the rapid removal of $CO_2$ from the blood by over-ventilation and partly by the fact, established by Edwards (1936) during the Expedition, that at really high altitudes even maximal work is accompanied by relatively low concentrations of lactic acid.

*Effects on the oxygen transport.* The general question of gas equilibria in the lungs at high altitude is discussed in this series by Dill, Christensen and Edwards (1936). Here it is only intended to draw certain physiological inferences from the data on the $O_2$ curves.

Except at sea level the data are insufficient for the construction of complete dissociation curves so that the curves are only defined precisely between about 40 and 80 per cent saturation. Below this range the curve has no great physiological significance in arterial blood and above it technical difficulties intervene. Though we have little direct evidence to justify the assumption that the general form of the curve above 80 per cent saturation is maintained at high altitude, points obtained for the arterial bloods as drawn fall more or less closely on the generalized dissociation curves.

In his discussion of the oxygen dissociation curve at high altitude, Barcroft has stressed the physiological importance of any adaptation which will increase the ease with which hemoglobin becomes oxygenated. Recently he (Barcroft, 1934) stated that increased affinity between Hb and $O_2$, such as he postulated from the Cerro de Pasco work, is not an unmitigated blessing at high altitude: "This shift in the dissociation curve [to the left] is of course prejudicial to the unloading of the blood and therefore prejudicial to the organism, it is a concession to the fact that oxygen must get into the hemoglobin before it can get out, but it tends to immobilize the organism" (1934, p. 222). If we pursue the reasoning on the

benefits or disadvantages to the organism of changed position of the $O_2$ dissociation curve, it is primarily with the tissues we must be concerned ultimately. We now know that fairly heavy work can be maintained for long periods in the face of relatively low arterial saturations and we suggest that, as the physiological range of the respiratory cycle moves downward on the dissociation curve, the completeness with which oxygen is unloaded to the tissues becomes increasingly important. In any case, the facts of the matter emerge when concrete data are analyzed. This is done in figure 6 for one case, viz., the mean for the 10 Expedition members at 'Quilcha, 5,340 meters.

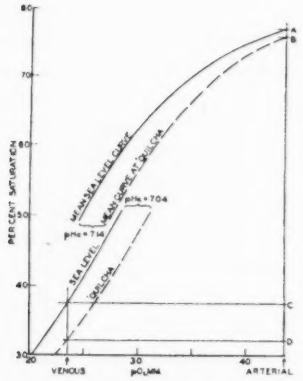


Fig. 6. An example of the effect on the amount of oxygen unloaded to the tissues of the observed shift of the oxygen dissociation curve. Mean from data from 'Quilcha, 5,340 meters. The broken curves are as found at 'Quilcha at the arterial $pH_c$ and at an approximate venous $pH_c$; the assumed difference in $pH_c$ between arterial and venous blood is more characteristic of moderate work than of rest, but this has little effect on the general result. The solid curves are from sea level for the same individuals at the same $pH_c$ values. Arterial $pO_2$ as found at the place, venous $pO_2$ approximate. The relative amount of oxygen unloaded to the tissues is shown by the length BD; had there been no change in the affinity of Hb for $O_2$, the $O_2$ unloaded would have been given by the length AC. The difference between BD and AC represents the gain to the tissues produced by the changed position of the curve.

It is unnecessary to elaborate on the significance of figure 6; the relative amount of oxygen unloaded to the tissues is given by the length of the line BD and what would have been unloaded at the same pH conditions, had there been no change in the curve from sea level, is given by the length AC. Had the curve shifted to the left, still less $O_2$ would have been unloaded. Table 4 presents the mean results of calculations of this sort for all our high altitude stations. To make conditions comparable a standard venous $O_2$ pressure of 25 mm. and a constant $HbO_2$ capacity of 20 vols. per cent are assumed. In the table no allowance is made for the change in $pH_c$ from arterial to venous blood; this does not affect the relative influence of change in the position of the curve.

The last column in table 4 gives the per cent $O_2$ gain to the tissues resulting from the observed shift in the curves. The reasons for the differences at different altitudes in the gain to the tissues are clear. At the lowest station the shift was very small and at the highest station, Punta, the arterial $pO_2$ is so low that the arterial blood is

represented on a part of the curve where the slope is approaching the venous slope. It should be pointed out that the gain to the tissues would be maintained down to very low venous $pO_2$ values, but that below about 7 mm. $pO_2$ it would tend to disappear and below about 3 mm. $pO_2$ the effect would be reversed for blood with an arterial saturation of less than 85 per cent.

It is not intended to stress unduly the physiological significance of the changed position of the curve. The residents exhibited it to a smaller degree than did our party, so it seems probable that it is in part only a temporary phenomenon, perhaps associated with the excess alkalinity of the blood in incomplete acclimatization. In any case, it acts in the direction of overcoming the effects of the excess alkalinity on the affinity of the

TABLE 4

*The theoretical effect of position of the dissociation curve on the relative amounts of oxygen which would be unloaded to the tissues at a venous $pO_2$ of 25 mm. by blood of 20 vols. per cent $HbO_2$ capacity previously equilibrated at the indicated arterial $O_2$ pressures*

Calculated from mean data for 10 Expedition members. No difference between arterial and venous $pH_c$ is assumed. Arterial $pO_2$ and $pH_c$ values as found at the respective stations.

| STATION | ALTITUDE, METERS | MEAN ARTERIAL $pO_2$ | MEAN ARTERIAL $pH_c$ | cc. $O_2$ UNLOADED PER L. BLOOD | | | | PER CENT $O_2$ GAIN TO TISSUES AT ART. $pH_c$ |
|---|---|---|---|---|---|---|---|---|
| | | | | Sea level curve | | Curve of place | | |
| | | | | At $pH_c$ = 7.10 | At $pH_c$ = art. | At $pH_c$ = 7.10 | At $pH_c$ = art. | |
| Chuquicamata | 2,810 | 60.1 | 7.13 | 85.6 | 81.4 | 85.4 | 80.4 | −1 |
| Ollagüe | 3,660 | 47.5 | 7.17 | 70.0 | 64.2 | 72.8 | 66.2 | +3 |
| Collahuasi | 4,700 | 43.5 | 7.16 | 62.6 | 58.0 | 67.0 | 61.4 | +6 |
| 'Quilcha | 5,340 | 43.1 | 7.14 | 61.8 | 57.2 | 66.0 | 64.3 | +12 |
| Punta | 6,140 | 34.4 | 7.13 | 40.0 | 36.6 | 38.0 | 40.0 | +9 |

Hb for $O_2$ and, from that point of view, may be considered to be a homoeostatic adjustment.

## SUMMARY

The position of the oxygen dissociation curve of whole arterial blood was studied on 10 men at 6 levels of altitude, from sea level to 6,140 meters, over a period of from 2 to 3½ months at high altitude.

At constant pH of the cells there was no tendency toward an increased affinity of hemoglobin for oxygen but instead there was a uniform tendency in the opposite direction, so that, on the average, at 6,140 meters 3.5 mm. $O_2$ pressure more than at sea level were required to produce half saturation.

The "physiological" dissociation curves were displaced to the left of sea

level up to about 14,000 feet; above that altitude the curves were displaced increasingly to the right of the sea level positions.

The theoretical significance of the observed changes in the fundamental affinity of Hb for $O_2$ is discussed and it is shown that they may be considered to be advantageous at high altitude in that they provide for an increased delivery of oxygen to the tissues at all except values for venous saturation close to zero and arterial saturations lower than about 65 per cent.

Dissociation curves on arterial blood drawn in work gave results similar to those obtained in basal rest.

The positions of the dissociation curves were determined for 11 long-time residents at altitudes up to 5,340 meters; 8 of these men were long accustomed to daily labor at 5,700 meters. Both at arterial $pH_c$ and at constant $pH_c$ these dissociation curves were, in general, within the normal limits for men at sea level; at constant $pH_c$ the average was perhaps slightly to the right.

Comparison of the residents with the Expedition members indicated that in both cases there is a tendency to achieve homoeostasis and that alteration in the position of the $O_2$ curve at high altitude may be an intermediate stage in final adaptation.

## REFERENCES


BARCROFT, J. The respiratory function of the blood. Part I. Lessons from high altitudes, Cambridge University Press, 1925.
Features in the architecture of physiological function. Cambridge University Press, 1934.

BARCROFT, J., BINGER, A. V. BOCK, DOGGART, FORBES, G. A. HARROP, J. C. MEAKINS AND A. C. REDFIELD. Phil. Trans. Roy. Soc. B **211:** 351, 1922.

BARCROFT, J., R. H. E. ELLIOTT, L. B. FLEXNER, F. G. HALL, W. HERKEL, E. F. MCCARTHY, T. MCCLURKIN AND M. TALAAT. J. Physiol. **83:** 192, 1934.

BRINKMAN, R. AND J. H. P. JONXIS. J. Physiol. **85:** 117, 1935.

BRINKMAN, R., A. WILDSCHUT AND A. WITTERMANS. J. Physiol. **80:** 377, 1933.

BROWN, W. E. L. AND A. V. HILL. Proc. Roy. Soc. B **94:** 297, 1923.

BUIKOV, K. M. AND E. E. MARTINSON. Arch. sci. biol. (U. S. S. R.) **33:** 147, 1933.

DILL, D. B., E. H. CHRISTENSEN AND H. T. EDWARDS. To be published. 1936.

DILL, D. B., H. T. EDWARDS, A. FÖLLING, S. A. OBERT, A. M. PAPPENHEIMER, JR. AND J. H. TALBOTT. J. Physiol. **71:** 47, 1931.

DILL, D. B., H. T. EDWARDS, M. FLORKIN AND R. W. CAMPBELL. J. Biol. Chem. **95:** 143, 1932.

DOUGLAS, C. G., J. S. HALDANE, Y. HENDERSON AND E. C. SCHNEIDER. Phil. Trans. Roy. Soc. (London) B **203:** 185, 1913.

EDWARDS, H. T. To be published. 1936.

FORBES, W. H., A. KEYS AND F. G. HALL. To be published. 1936.

GEIGER, A. Proc. Roy. Soc. B **107:** 368, 1931.

HALDANE, J. S. AND J. G. PRIESTLEY. Respiration. Yale University Press, 1935.

HALL, F. G. J. Physiol. **80:** 33, 1934a.
J. Physiol. **80:** 502, 1934b.
J. Physiol. **83:** 222, 1934c.
To be published. 1936.
HENDERSON, L. J. Blood. Yale University Press, 1928.
HENDERSON, L. J., D. B. DILL, H. T. EDWARDS AND W. O. P. MORGAN. J. Biol. Chem. **90:** 697, 1931.
KEYS, A. Geog. Rev. To be published. June, 1936.
KEYS, A., B. H. C. MATTHEWS, AND W. H. FORBES. To be published, 1936.
LITARCZEK, G., H. AUBERT AND I. COSMULESCO. Compt.-rend. soc. biol., **106:** 973, 1933.
LOONEY, J. M. AND H. M. CHILDS. J. Biol. Chem. **104:** 53, 1934.
MCCARTHY, E. F. J. Physiol. **80:** 206, 1933.
MACELA, I. AND A. SELIŠKAR. J. Physiol. **60:** 428, 1925.
RUBOWITZ, M. Biochem. Ztschr. **266:** 190, 1933.
STADIE, W. C. AND E. R. HAWES. J. Biol. Chem. **77:** 265, 1928.
TALBOTT, J. H. AND W. V. CONSOLAZIO. To be published, 1936.
TALBOTT, J. H. To be published in Haematology, 1936.
VAN SLYKE, D. D., J. SENDROY, A. B. HASTINGS AND J. M. NEILL. J. Biol. Chem. **78:** 765, 1928.

# SOME AFFERENT NERVES PRODUCING REFLEX RESPONSES OF THE NICTITATING MEMBRANE

G. H. ACHESON, A. ROSENBLUETH AND P. F. PARTINGTON

*From the Department of Physiology in the Harvard Medical School*

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A previous communication from this Laboratory (Rosenblueth and Schwartz, 1935) described reflex contractions and relaxations of the intrinsic smooth musculature in the nictitating membrane (n.m.) of the cat. A qualitative survey of some of the afferents capable of eliciting these reflexes was deemed desirable as a preliminary step for future quantitative studies. The present report deals with this survey.

METHODS. Cats were used. After etherization, urethane (1 to 1.5 gram per kgm.) was injected intravenously.

Isotonic responses of the n.m. were recorded by a lever exerting a tension of approximately 2 grams and affording a 10- to 15-fold magnification. The blood pressure (b.p.) was recorded from a carotid or femoral artery by means of a mercury manometer. The b.p. was in all cases satisfactory, higher than 120 mm. Hg. The respiratory movements were recorded through a Marey pneumograph or by connecting the tracheal cannula to a writing tambour through a T-tube, the other branch of which was open.

The adrenal glands were routinely ligated. Curare was employed to eliminate possible confusing eye movements when the responses of the n.m. were small or doubtful.

Stimulation of the afferent nerves, crushed or cut peripherally, was effected through shielded electrodes. The stimuli were either shocks from a Harvard induction coil with 5 volts in the primary circuit or short (about 1 $\sigma$) rectangular waves from a "multivibrator" circuit. Spread of current to neighboring nerves was avoided by cutting them centrally to the site of stimulation. The rôle of the afferent nerve stimulated was further ascertained, when deemed necessary, by crushing it centrally to the electrodes and observing whether the reflex disappeared.

The vagi and depressor nerves were sometimes cut and the carotids denervated in order to enhance the reflex contractions of the n.m. (Rosenblueth and Schwartz, *loc. cit.*) and the rises of b.p.

RESULTS. I. *Cutaneous afferents.* The saphenous nerve was selected as a typical cutaneous afferent. Stimulation of this nerve readily evokes reflex responses of the n.m. These responses are a function of both the

frequency and the intensity of stimulation. Weak shocks at low frequencies favor relaxations of the n.m. (fig. 1A), while strong shocks at high frequencies favor contractions (fig. 2A). Reflex relaxations are as a rule difficult to obtain; a tonic background is necessary and often it is not sufficiently strong.

With strong shocks even slow frequencies (below 2 per second) evoke contractions of the n.m. Indeed, sometimes the n.m. contracts in response to single afferent volleys (fig. 1B). On the b.p. slow frequencies (below 10 per second), even with strong shocks, as a rule yield a fall (fig. 1C). If a relatively high frequency (e.g., 50 per second) of stimulation is applied with increasing intensities, the n.m. usually contracts with weak shocks that induce a fall of b.p. There is thus a difference between the two

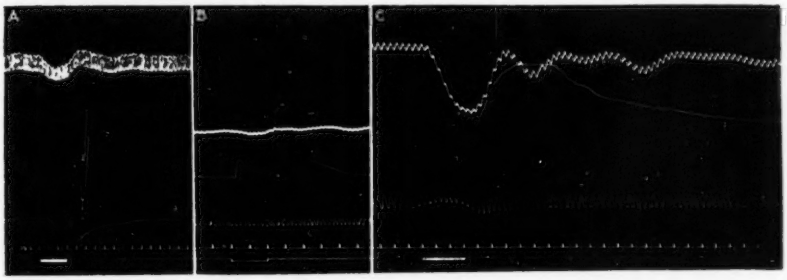


Fig. 1. Upper records: b.p.; middle records: n.m.; lower records: respiration. Time signal: 5 second intervals. At lower signal central stimulation (induction shocks) of the saphenous nerve.

A. Curare. Frequency: 6 per second; coil distance: 12 cm. B. One make and one break; coil distance: 8 cm. C. Vagi and depressors cut; carotids denervated. Coil distance: 6 cm.; frequency: 2.6 per second.

reflexes, the change from − to + with increasing frequencies and intensities of stimulation occurring sooner for the n.m. than for the b.p.

Rebounds—i.e., sudden changes at the end of stimulation that are not explainable merely as the subsidence of the reflex response—were often observed in both b.p. and n.m. responses (figs. 1C and 2A). Again the two systems differed in that while the rebounds of the n.m. were usually excitatory, those in the b.p. were as a rule inhibitory. The most striking rebounds of the n.m. were obtained with strong stimuli at low frequencies (fig. 1C).

Prolonged (several minutes) reflex after-discharges to the n.m. were frequent. The duration of these after-discharges increased usually with increasing intensities and frequencies.

II. *Muscular afferents.* The hamstring and the hypoglossal nerves were studied.

The reflexes from the hamstring nerves were qualitatively similar to those obtained from the saphenous (fig. 2). Some quantitative differences will be described later (section VII).

Stimulation of the hypoglossal nerve elicited only slight contractions of the n.m. Rises of b.p. were easily obtained (fig. 3). Falls of b.p. were only evoked by strong shocks at low frequencies.

III. *Cornea.* The stimulating electrodes were applied by hand on the surface. There being a possibility of spread of current to neighboring regions the results from strong stimuli must be taken guardedly.

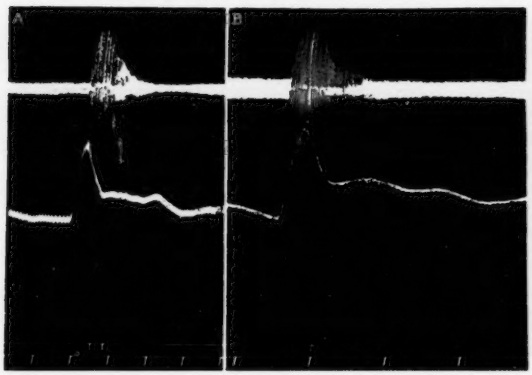


Fig. 2

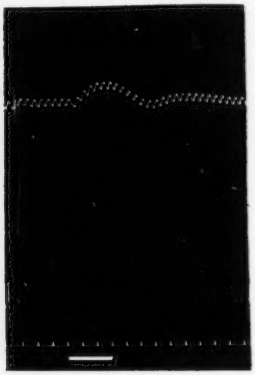

Fig. 3

Fig. 2. Records: upper, respiration; middle, b.p.; lower, n.m. Time signal: 30 second intervals. Between upper signals stimuli applied; frequency: 50 per second; voltage: 17.

A. Central stimulation of the saphenous nerve. B. Central stimulation of the hamstring nerves.

Fig. 3. Vagi and depressors cut; carotids denervated. Records: upper, b.p.; middle, n.m.; lower, respiration. Time signal: 5 second intervals. At lower signal central stimulation of the hypoglossal nerve. Coil distance: 8 cm.

Moderate stimuli yielded only rises of b.p. at all frequencies (cf. Gerard, 1923), while the n.m. usually relaxed. The respiratory effects were inconsistent. All responses were small, the b.p. seldom rising more than 10 mm. of Hg. Dilatation of the pupil (Gerard, *loc. cit.*) only occurred with very strong stimuli, possibly by spread of current.

IV. *Visceral afferents in sympathetic nerves.* The splanchnic, hypogastric and hepatic nerves were investigated, and also the cardiac branch of the stellate ganglion. No relaxations of the n.m. were observed. Contractions, on the other hand, were obtained readily (figs. 4, 5 and 6).

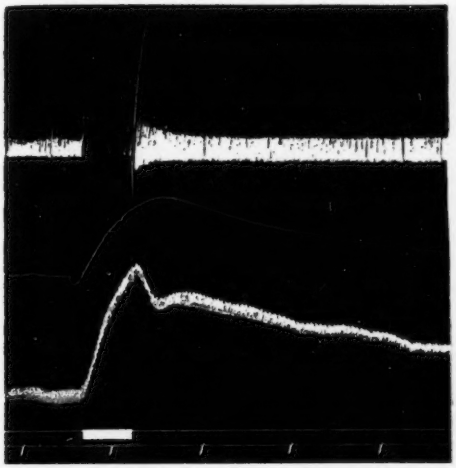

Fig. 4

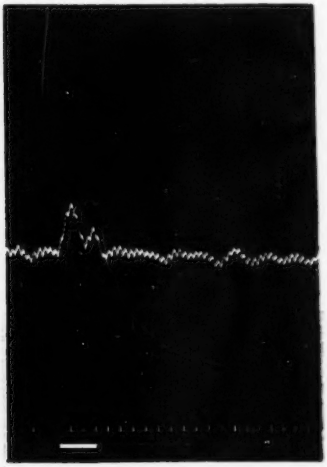

Fig. 5

Fig. 4. Records: upper, respiration; middle, n.m.; lower, b.p. Time signal: 30 second intervals. At upper signal central stimulation of the major splanchnic nerve. Coil distance: 10 cm.

Fig. 5. Curare. Records: upper, b.p.; lower, n.m. Time signal: 5 second intervals. At lower signal central stimulation of the cardiac branch of the stellate ganglion; coil distance: 6 cm.

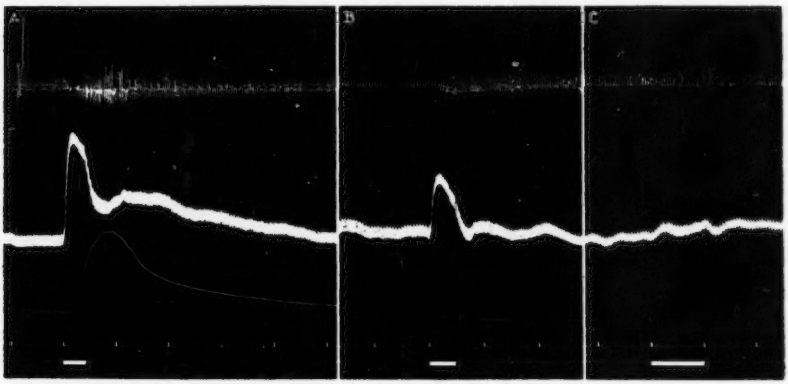


Fig. 6. Records: upper, respiration; middle, b.p.; lower, n.m. Time signal: 30 second intervals. At lower signals central stimulation of the hepatic nerves; coil distance: 8 cm.

A. Splanchnics intact. B. After cutting the major splanchnics. C. After cutting the minor splanchnics.

Excitatory rebounds appeared with all afferents mentioned, except the hepatic nerves.

Weak and slow stimuli applied to the splanchnics or the cardiac nerves induced falls of b.p. Stimulation of the hypogastrics or splanchnics with strong, rapid shocks often produced a rise of b.p. succeeded at the end of stimulation by a negative rebound, the b.p. falling below the basal level, and a prolonged negative after-effect (cf. Rosenblueth and Schwartz, *loc. cit.*, fig. 8D).

The hepatic afferents appear to contain only fibers inducing excitatory autonomic effects. Severing the major splanchnics practically abolished

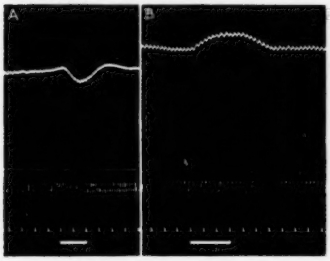


Fig. 7

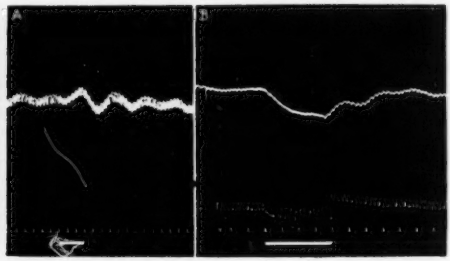


Fig. 8

**Fig. 7.** Records: upper, b.p.; middle, n.m.; lower, respiration. Time signal: 5 second intervals. At lower signals central stimulation of the cervical sympathetic.

A. Coil distance: 10 cm. B. Vagi and depressors cut; carotids denervated. Coil distance: 8 cm.

**Fig. 8A.** Curare. Records: upper, b.p.; lower, n.m. Time signal: 5 second intervals. At lower signal central stimulation of a pelvic nerve; coil distance: 10 cm.

B. Records: upper, b.p.; middle, n.m.; lower, respiration. Time signal: 5 second intervals. At lower signal central stimulation of a pelvic nerve; coil distance: 10 cm.

the responses of the n.m., while b.p. and respiratory changes still occurred (fig. 6B). If the minor splanchnics were then cut all reflex effects disappeared (fig. 6C). The vagi, therefore, apparently do not carry any afferent fibers from the liver which elicit any of the reflexes recorded.

V. *Cervical sympathetic.* On the side opposite that of the recording n.m. the vagus and depressor nerves were cut below and above the site of application of the electrodes on the cervical sympathetic. This nerve was cut peripherally near the superior cervical ganglion, dissected for about two inches and held separated from any other tissues while the stimulating electrodes were applied by hand.

The results on the b.p. were slight but definite (9 cases out of 11).

There were primarily rises, occasionally falls (fig. 7). The responses of the n.m. and respiration were questionable. These effects disappeared on crushing the nerve centrally.

VI. *Afferents in parasympathetic nerves.* The reflex relaxations of the n.m. that may be obtained on stimulation of the vagus in the neck have been previously described (Rosenblueth and Schwartz, *loc. cit.*). The pelvic nerves were studied. Both contractions and relaxations of the n.m. were obtained. In the b.p., falls were common, occurring in response to weak stimuli at tetanizing frequency or to stronger shocks at low frequencies. Rises of b.p. were also observed. Figure 8 illustrates typical instances.

VII. *The respiratory responses.* Reflex respiratory changes were elicited from all the afferents studied. These changes involved not only the rate, but also the depth of the respiratory excursions. The variations of rate and depth were independent of each other, i.e., the rate was unchanged, increased or decreased, while the depth could either increase or decrease. Prolonged after-effects were frequently noted, especially after strong stimuli (figs. 2 and 6).

Respiration is frequently irregular under urethane anesthesia. Inspiratory pauses are common (apneusis). The reflex effects often were attended by a regularization of the respiratory movements. A heating pad was used to maintain a normal body temperature. Panting was frequently recorded when the heating pad was applied (figs. 2 and 4), even after section of the vagi, and often with the rectal temperature normal, or lower than normal. The afferent stimuli commonly produced a slower respiration, but still more rapid than normal (e.g., from about 200 per minute during panting to about 110 during the stimulus, fig. 2B).

It is interesting that stimulation of the hepatic nerves, which had uniform excitatory effects on the n.m. and b.p. (section IV), elicited mixed respiratory responses, e.g., slowing succeeded shortly by acceleration (fig. 6A).

Stimulation of the pelvic nerves (sometimes also of the hypogastrics) usually elicited marked inspiratory effects, frequently associated with slowing of the rate (fig. 8B). From the splanchnic nerves inhibition of the respiration was prominent (fig. 4).

VIII. *Quantitative differences between afferents.* The simultaneous records of n.m. and b.p. changes permit quantitative comparisons to be made between different afferents. Thus, stimuli at a constant frequency separately applied to the hamstring or saphenous and the hypogastric nerves, adjusted in their intensity so that equal rises of b.p. were obtained, produced usually greater contractions of the n.m. with the hamstrings or saphenous than with the hypogastric stimulation. Figure 2 illustrates such a comparison between the hamstrings and the saphenous. The re-

sults of these comparisons were not entirely consistent for certain nerves—i.e., apparently greater effects on the n.m. were recorded in some cases from the hamstrings than from the saphenous (fig. 2), and in other cases the reverse occurred. Some differences, however, were consistent. The following series represents a gradation of the relative efficiency of some of the afferents studied in eliciting b.p. rises (n.m. constant): splanchnics or hypogastrics > cardiac nerves > saphenous or hamstrings. The reverse order is therefore descriptive of the excitatory effects in the n.m.

Discussion. That practically all afferent nerves may induce reflex changes of b.p., heart rate and respiration is perhaps not surprising if we consider that respiratory and circulatory adjustments are indispensable in any reaction of the organism. It is, however, surprising that all the various afferents tested were capable of eliciting reflex changes of the n.m. A purposive interpretation of each of these responses would be idle. It is reasonable to correlate them with Cannon's (1930) principle of the tendency of the sympathetic nervous system to react as a whole. This reaction is, however, not invariably the same, as shown by the differential effects of the several afferents on the n.m. and the b.p. (section VIII) and by the indications of "local signs" to be discussed below.

The majority of the afferents studied do not require specific comments. The reactions obtained from the hypoglossal and the cervical sympathetic deserve special mention. The hypoglossal has been frequently supposed to be a purely motor nerve, devoid of afferents. Langworthy (1924) however, presented anatomical evidence that there is an afferent component in most cats. Our results (fig. 3) corroborate this evidence.

The cervical sympathetic has also been commonly described as lacking an afferent component. The test has usually been that of "pain" reactions in lightly anesthetized or unanesthetized animals. Such tests yielded negative results in cats (Cleveland, 1932; Davis and Pollock, 1932; B. Cannon, 1933). It is possible that the afferents responsible for the responses recorded in the present study (fig. 7) are not "pain" fibers, or else that their threshold is high and sufficiently strong stimuli were not used in the negative experiments. Reflex changes of b.p. have also been investigated (Langley, 1892; Ranson and Billingsley, 1918), likewise with negative results. These experiments, however, were performed under A. C. E. anesthesia, which might depress the slight effects recorded here under urethane.

When, on stimulation of an afferent nerve, responses of opposite sign are encountered—i.e., rises or falls of b.p., contractions or relaxations of the n.m.—it is probable that the nerve is mixed, containing both excitatory and inhibitory fibers for the response involved. Similarly, rebounds denote the probable mixed character of the afferent that evokes them (Sherrington and Sowton, 1911). With the exception of the afferents

from the cornea (section III) and the hepatic nerves (fig. 6), all the nevers studied here gave either responses of opposite sign, or rebounds, or both, as well on the n.m. as on the b.p. The unmixed nature of the afferents from the cornea is generally accepted (cf. Gerard, *loc. cit.*). As regards the hepatic nerves, it appears plausible that the only afferents capable of inducing b.p. rises or n.m. contractions may be "pain" fibers from the organ. The interesting feature is noticeable, however, that the fibers in the minor splanchnics elicit b.p. rises, but practically no contractions of the n.m. (fig. 6B). Whether the mixed effects of the hepatic nerves on the respiration (fig. 6A) are due to complexity of the afferents or to the known complexity of the respiratory center may not be decided at present.

The majority of the reflex responses recorded bear no indications of a local sign—i.e, of a specific spatial correlation between the site of origin of the afferents stimulated and the reflex evoked. Probably a local sign may account for the failure of the "pain" fibers from the cornea to evoke contractions of the n.m. The purposive interpretation of such a local sign is obvious: when an irritating body stimulates the cornea the n.m. sweeps outward on contraction of the extrinsic skeletal musculature; a contraction of the intrinsic smooth muscle would oppose this motion.

A recognition of "pain" afferents from the reflex responses evoked in anesthetized animals would be desirable. The present data lead to discouraging conclusions in this respect. Thus, hyperpnea has been often selected as an indicator of pain responses. Other afferents than "pain" fibers, however, may elicit hyperpnea, e.g., the "heat" afferents. Furthermore, "pain" fibers may bear a local sign, which may oppose hyperpnea; thus, the typical respiratory effects evoked by certain abdominal visceral afferents (figs. 4 and 8B) may well be interpreted as indicative of such a local sign. Generalized sympathetic hyperactivity could also be suggested as indicative of "pain" responses. Objections similar to those mentioned for hyperpnea oppose this suggestion. A discrimination of the functions of other afferents than "pain" fibers in the reflexes recorded might be obtained from application of "physiological" stimuli: e.g., heat, cold, etc. (cf. panting, p. 313).

Although little is known about the composition of the majority of the nerves studied, the method employed, of comparing their relative effects on the b.p. and the n.m. (section VIII), reveals quantitative differences of significance. From these differences the following conclusions appear legitimate: diverse sympathetic reflex responses vary independently; hence, the several afferents studied make independent connections in the central nervous system. These conclusions lead further to the view that each component in each afferent belongs to discrete reflex arcs and that possibly the only overlap of these several arcs occurs at the final common paths.

SUMMARY

In cats under urethane anesthesia reflex responses in the nictitating membrane and blood pressure may be elicited by afferent stimulation of the following nerves: saphenous (figs. 1 and 2), hamstring (fig. 2), hypoglossal (fig. 3), splanchnic (fig. 4), hypogastric (section IV), hepatic (fig. 6), cervical sympathetic (fig. 7), pelvic (fig. 8), and also the cardiac branch of the stellate ganglion (fig. 5). Stimulation of the cornea elicits rises of blood pressure but has minimal effects on the nictitating membrane (section III).

Quantitative differences between the several afferents as regards their relative effects on the nictitating membrane and the blood pressure were recorded (section VIII).

These reflexes are discussed in relation to the following problems: purpose (p. 314), mixed composition of the afferents (p. 314), "pain" afferents (p. 315), and central connections (p. 315)

REFERENCES

CANNON, B. This Journal **105**: 366, 1933.
CANNON, W. B. Linacre Lecture. Lancet, p. 1109, 1930.
CLEVELAND, D. A. J. Comp. Neurol. **54**: 35, 1932.
DAVIS, L. AND L. G. POLLOCK. Arch. Neurol. Psychiat. **27**: 282, 1932.
GERARD, M. W. Ibid. **9**: 306, 1923.
LANGLEY, J. N. Phil. Trans. Roy. Soc. London **183**: 85, 1892.
LANGWORTHY, O. J. Comp. Neurol. **36**: 273, 1924.
RANSON, S. W. AND P. R. BILLINGSLEY. Ibid. **29**: 312, 1918.
ROSENBLUETH, A. AND H. G. SCHWARTZ. This Journal **112**: 422, 1935.
SHERRINGTON, C. S. AND S. C. M. SOWTON. Proc. Roy. Soc., B, **83**: 435, 1911.

# ON THE ACTION OF HEPARIN AND ITS RELATION TO THROMBOPLASTIN

ARMAND J. QUICK

*From the Department of Pharmacology, Marquette University School of Medicine, Milwaukee*

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Much uncertainty remains regarding the action of heparin in preventing the coagulation of blood. Howell, who with Holt (1) first prepared this substance, and who has intensively studied it (2, 3) believes that it is essentially an antiprothrombin normally existing in the blood combined with prothrombin. He postulates that it can be removed from this combination by the tissue factor (cephalin-protein complex) and that following this neutralization, the liberated prothrombin reacts directly with calcium thereby changing to active thrombin. Howell, moreover, observed that while heparin when mixed with purified fibrinogen and thrombin exhibited no antithrombic action, it markedly inhibited clotting when added to serum or plasma. This led him to conclude that heparin itself is not an antithrombin, but causes the production of a true antithrombin by reacting with a thermolabile substance occurring in the blood. Recently Mellanby (4) has questioned the view that heparin is an antiprothrombin, stating that he could find no evidence that this substance had any inhibitory action on the conversion of prothrombin to thrombin. He confirmed Howell's finding that heparin is inactive as an anticoagulant when thrombin is allowed to react with fibrinogen, but found that this was only true in the absence of neutral salts. He concluded that heparin itself is an antithrombin but requires neutral salts for its activity. Schmitz and Kuhl (5) recently have come to the same conclusion. Mellanby is, however, in accord with Howell in his belief that the antithrombic action of heparin is annulled by thromboplastin. Likewise Waldschmidt-Leitz (6) has suggested that the anticlotting action of heparin may be due to its property of neutralizing thromboplastin.

The need for further study of the action of heparin is clearly indicated by these conflicting opinions. In such an investigation it is essential, as Howell (7) has emphatically pointed out, that the several reagents which enter into the process of coagulation, namely, fibrinogen, thrombin, prothrombin, antiprothrombin, the tissue factor, etc., be prepared in a purified and standardized form. While such a rigid requirement can only

be met in a relative manner, an effort was made in the present study to use only preparations having a high degree of potency and being as free as possible from objectional impurities, and to employ methods so standardized that consistent quantitative results could be obtained. Due cognizance, however, was taken of the probability that the interaction of purified reagents in vitro may not represent the complete or even the correct picture of the normal physiological clotting of blood. Moreover, care was taken to specify in the recording of every experiment, the species from which the material was obtained, since it was observed that a marked difference in the clotting factors is found in the bloods of different animals. The significance of these variations is at present difficult to evaluate, but the fact that they do exist demands great caution in the interpretation of results.

PREPARATION OF REAGENTS. *Thrombin.* The method of Eagle (8) was found highly satisfactory. The preparation was as follows: 10 cc. of citrated or oxalated human plasma were diluted with 100 cc. of cold distilled water, and carbon dioxide bubbled through the solution for 5 minutes. The precipitate which formed was removed by centrifuging, and after the complete removal of the supernatant liquid, was dissolved in 10 cc. of 0.85 per cent sodium chloride solution. Enough sodium bicarbonate was added to bring the solution to pH 7.0. After warming the solution to 37°C., 0.5 cc. of 0.1M. calcium chloride was added. The fibrin which formed in about 2 minutes was removed by wrapping it around a glass rod. The solution thus prepared not only had a high but also a remarkably constant degree of thrombic potency. The uniform yield of thrombin obtained using a series of normal human plasmas further demonstrates the noteworthy constancy of the clotting factors in human blood. Nevertheless, it should be mentioned that carbon dioxide does not precipitate all the prothrombin, and therefore the method cannot be employed to determine the total thrombin potentially obtainable from plasma.

*Fibrinogen.* This serum protein can be removed from plasma either by adding 1 part of saturated ammonium sulfate to 3 parts of plasma, or 1.2 parts of saturated sodium chloride to 1 part of plasma. By using human plasma and treating the fibrinogen solution with a small amount of aluminum hydroxide, a preparation can readily be obtained which is free from prothrombin. In a former study the writer (9) used ammonium sulfate as the precipitating agent; but for studying the action of heparin on thrombin, fibrinogen obtained from plasma by means of saturated sodium chloride was found more satisfactory. In studying the effect of different concentrations of electrolytes on clotting time, no elaborate means of purification was employed; merely 1 volume of plasma was mixed with 1.2 volumes of saturated sodium chloride solution and the precipitate

which formed was tightly packed by rapid centrifugation. The supernatant liquid was removed, and the precipitate washed by rinsing the centrifuge tube with distilled water. The fibrinogen thus obtained was dissolved in a solvent containing the desired concentration of electrolyte.

*Dialyzed plasma.* By using a slightly modified Simms' concentrating dialyzer (10), plasma can be dialyzed not only more rapidly and efficiently but also without change of volume even against distilled water.

*Prothrombin-free plasma (alumina plasma).* By mixing 1 cc. of oxalated human plasma with 0.1 cc. of a thick aluminum hydroxide cream, and incubating for 15 minutes at 37°C., a plasma is obtained which after the aluminum hydroxide is removed will no longer clot on recalcification even when excess thromboplastin is present.

*Heparin.* The commercial preparation of Hynson, Westcott, and Dunning was used. Although more highly purified preparations had a greater degree of activity, the essential reactions were found to remain unchanged. A sample of heparin kindly furnished by Charles and Scott was found to react almost exactly as the product obtained from Hynson, Westcott, and Dunning, although their preparation failed to give a positive naphthoresorcinol test which is strongly positive in the commercial product. It was considered desirable to use the heparin of Hynson, Westcott, and Dunning which is made in accordance with the directions of Howell, whose earlier studies were made on a similar product. Furthermore many subsequent investigators including Mellanby have likewise employed this preparation. No evidence was found to suggest that any of its impurities interfered in the reactions studied, and it seems justifiable to assume that the product contained the active principle. The utter lack of agreement in regard to the chemical composition of such highly purified preparations of heparin as recently described by Charles and Scott (11), by Schmitz and Fischer (12), and by Jorpes (13) make it difficult to accept any one as constituting the pure active principle.

*Thromboplastin.* Rabbit brain served as the source of thromboplastin. The preparation and properties of this agent were recently described (9). No product was used unless it showed maximum activity, i.e., clotted oxalated rabbit plasma in 7 to 9 seconds and human plasma in 16 to 18 seconds when recalcified in accordance with the author's prothrombin test.

In the present study, which had for its object securing further information concerning the action of heparin and its relation to thromboplastin, 4 observations will be presented.

*1. The rate of clotting is directly proportional to the concentration of thrombin, and is little influenced by the source or concentration of fibrinogen, but is retarded by increasing concentrations of sodium chloride.* On studying the action of thrombin on fibrinogen, it was found that the coagulation time is inversely proportional to the concentration of thrombin, or in

other words, a direct linear ratio appears to exist between the speed of clotting and the amount of active thrombin as seen in figure 1. A number of the earlier investigators including Ducleaux and Arthus (14), Rettger (15), and others noted this relationship. By using snake venom as a source of thrombin, Martin (16), and recently Barratt (17), were able to

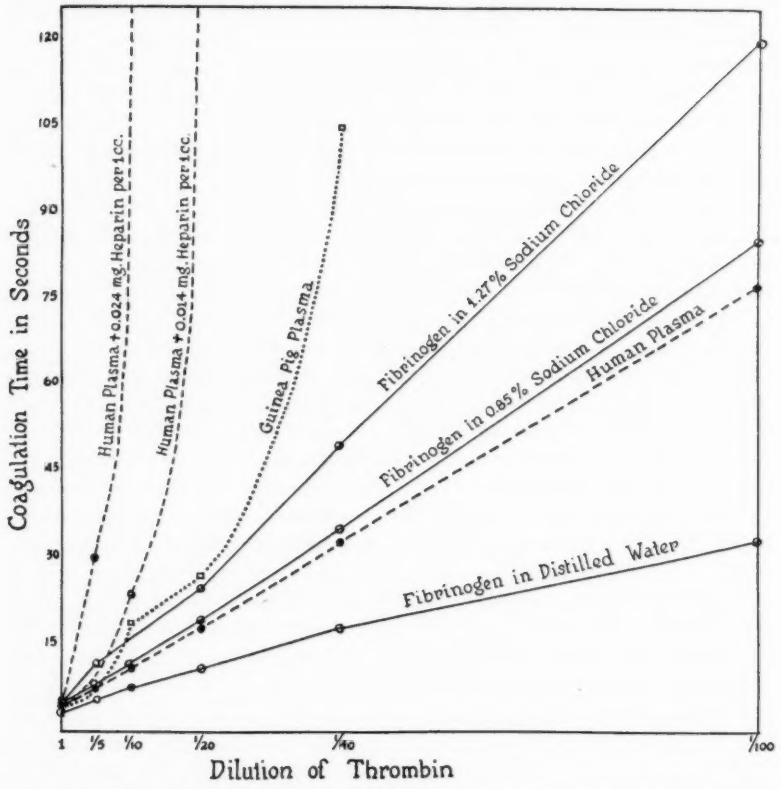


Fig. 1. The relation of the coagulation time to the concentration of thrombin. The coagulation time was determined by mixing 0.2 cc. of plasma or fibrinogen solution with 0.1 cc. of thrombin solution.

show that the relationship between the concentration of thrombin and the clotting time can be expressed by a simple linear equation. Interestingly, the source and concentration of fibrinogen seem to have little influence on the clotting time, a finding which is in agreement with the generally accepted view that fibrinogen is non-species specific and is a passive component in the coagulation process. Even oxalated frog plasma when

treated with thrombin derived from human blood clotted in 7 seconds (0.2 cc. of frog plasma + 0.1 cc. thrombin). A series of fibrinogen solutions varying from 1.3 to 0.1 per cent, a range greater than ever occurring in the blood except in extreme pathological conditions, clotted with equal rapidity when mixed with a constant quantity of thrombin (table 1).

It is significant that the linear relationship between the clotting time and the concentration of thrombin was found to hold for oxalated plasma

TABLE 1

*The effect of the concentration of fibrinogen on clotting time*

| | | | | |
|---|---|---|---|---|
| Concentration of fibrinogen, per cent | 0.1 | 0.5 | 1.0 | 1.3 |
| Clotting time,* seconds | 10 | 9 | 9 | 9 |

* 0.2 cc. of fibrinogen (dissolved in normal saline) + 0.1 cc. thrombin.

TABLE 2

*The effect of the concentration of thrombin on the clotting time of fibrinogen solutions, and of plasmas from different animals*

| | CLOTTING TIME IN SECONDS† | | | | | |
|---|---|---|---|---|---|---|
| Concentration of thrombin* | 1 | 1/5 | 1/10 | 1/20 | 1/40 | 1/100 |
| Fibrinogen (human) dissolved in distilled water | 3.5 | 6 | 8 | 11 | 18 | 33 |
| Fibrinogen (guinea pig) dissolved in distilled water | 4 | 7 | 9 | 11 | 21 | 40 |
| Fibrinogen (dog) dissolved in approximately 0.85 per cent saline | 4.5 | 9 | 12 | 19 | 35 | 85 |
| Fibrinogen (dog) dissolved in approximately 1.27 per cent saline | 5 | 12 | 17 | 25 | 50 | 120 |
| Rabbit plasma | 4 | 7.5 | 12 | 17 | 33 | 77 |
| Dog plasma | 4 | 8 | 13 | 17 | 33 | 80 |
| Human plasma | 4 | 7.5 | 11.5 | 18 | 33 | 77 |
| Human plasma treated with aluminum hydroxide | 4 | 7.5 | 11.5 | 17 | 32 | 77 |
| Guinea pig plasma | 5 | 8 | 19 | 27 | 105 | No clot |

* Dilutions of thrombin were made with distilled water.

† The clotting time was determined by mixing 0.2 cc. of the fibrinogen solution or plasma with 0.1 cc. of the thrombin solution. All determinations made at 37°C.

as well as for purified fibrinogen solutions. Thus the clotting times of human, dog, and rabbit plasma, obtained with progressive dilutions of thrombin, corresponded to the values found using a solution of fibrinogen, as shown in table 2. Guinea pig plasma, however, failed to conform to the behavior of the other plasmas; with higher dilutions of thrombin its clotting time increased much more rapidly than would be expected if a direct proportionality existed between the speed of coagulation and

the concentration of thrombin. This peculiar behavior must be ascribed to a constituent in the plasma other than fibrinogen, since the latter when isolated and purified behaves the same as fibrinogen obtained from other species. It seems fairly probable that guinea pig plasma contains a trace of an antithrombin which can only manifest itself when the quantity of thrombin added is sufficiently small to allow complete neutralization. The direct proportionality found between the concentration of thrombin and the speed of coagulation of human, rabbit and dog plasma, strongly suggests that these plasmas contain no demonstrable amounts of antithrombin. Although the discussion of the action of heparin will be postponed until later, it should be pointed out that even an exceedingly small amount of this substance added to plasma will destroy the straight line proportionality between the clotting time and the concentration of thrombin as seen in figure 1.

Attention should be directed to the finding that oxalated plasma treated with aluminum hydroxide for the removal of prothrombin responded to thrombin exactly the same as untreated plasma. This can be taken as further evidence that aluminum hydroxide causes no essential change in plasma other than removing prothrombin.

Clotting is definitely influenced by the concentration of electrolytes. Fibrinogen dissolved in distilled water is relatively unstable, and is therefore rather difficult to work with. Such a solution responds promptly even to high dilutions of thrombin as can be seen in figure 1. The addition of sodium chloride as well as of other electrolytes stabilizes fibrinogen and retards its clotting by thrombin. Nevertheless, the clotting times of fibrinogen dissolved in solutions of a neutral salt when plotted against the concentrations of thrombin will give a straight line, in contrast to the sharp upward curve found when antithrombin is present.

On examining the results presented in figure 1, it can readily be seen that the relation of the clotting time to the concentration of thrombin can be expressed by the simple equation: $t = k\frac{1}{y}$ ($t$ = clotting time; $y$ = concentration of thrombin). The constant, $k$, is dependent upon the electrolyte concentration. It decreases with increasing concentrations of ionized neutral salts. For normal plasma its value is approximately 1. Obviously, the equation only holds true when the temperature and the hydrogen ion concentration are kept constant. The equation is of practical value since it can be applied to the clotting of plasma provided antithrombin is absent. As a matter of fact, the curve obtained by plotting coagulation time against thrombin concentrations may become a useful method for determining the absence or presence of antithrombin. It has been found by this means that dog blood which has been rendered incoagulable by the injection of peptone contains much antithrombin. The results of this study will be reported later.

*2. The coagulation of oxalated plasma by thrombin is progressively delayed and eventually arrested by increasing quantities of heparin, but in plasma dialyzed against distilled water and in solutions of purified fibrinogen, heparin has little anticoagulative action.* From the results presented in figure 1, it can be seen that the direct relationship between the clotting time and the concentration of thrombin is immediately destroyed by adding heparin to plasma, since dilute solutions of thrombin become neutralized and thus can no longer produce clotting. Similarly, by adding increasing amounts of heparin to plasma, the clotting time induced by a fixed amount of thrombin is progressively protracted, and ultimately a concentration can be reached which completely stops coagulation as seen in table 3. By employing a stronger solution of thrombin, more heparin is required to inhibit clotting. There is obviously a direct and fairly quantitative re-

TABLE 3

*The inhibitory action of increasing concentrations of heparin on the clotting of plasma by thrombin*

| Concentration of heparin in plasma (mgm. per 1 cc. of plasma) | 0.00 | 0.08 | 0.16 | 0.24 | 0.32 | 0.40 | 0.60 |
|---|---|---|---|---|---|---|---|
| Thrombin (½ S.).† Clotting time,* seconds | 5.5 | 34 | 80 | 150 | 660 | No clot | No clot |
| Thrombin (F. S.).‡ Clotting time, seconds | 4 | 9 | 15 | 28 | 44 | 80 | No clot |

* 0.2 cc. of plasma (containing heparin) + 0.1 cc. thrombin.

† (½ S.) = half strength, thrombin solution diluted with an equal volume of distilled water.

‡ (F. S.) = full strength.

lationship between the amount of heparin and the quantity of thrombin neutralized.

To determine whether heparin is itself an antithrombin as Mellanby maintains, or whether it gives rise to an anticoagulating agent by reacting with a thermolabile substance in the blood, some of the important experiments recently reported by Mellanby (4) were repeated. His significant observation that heparin loses its anticlotting power in plasma dialyzed against distilled water was confirmed, as seen in table 4. Rabbit plasma is especially well suited for this demonstration. By adding sufficient sodium chloride to approximate the electrolyte concentration of normal plasma, heparin again becomes effective, showing that its inhibitory action on clotting requires the presence of neutral salts. The crucial experiment for determining whether heparin per se is an antithrombin consists in studying its effectiveness in preventing the coagulation of purified fibrinogen by thrombin. By using ammonium sulfate for precipitating

fibrinogen, a preparation was obtained which when mixed with heparin showed a distinct delay in clotting compared with a control containing the same amount of thrombin but no heparin (table 5). Fibrinogen isolated from plasma by precipitation with half saturated sodium chloride, following essentially the classical method of Hammarsten, gave different results. Such a preparation clotted almost as promptly in the presence as in the absence of heparin. Even though one added a relatively large amount of heparin, no unmistakable delay in clotting could be demonstrated unless one employed a highly diluted solution of thrombin. The fact that

TABLE 4

*The effectiveness of heparin as an anticoagulant in normal plasma, plasma dialyzed against distilled water, and dialyzed plasma with sodium chloride restored*

| | | | | | | |
|---|---|---|---|---|---|---|
| Oxalated rabbit plasma: | | | | | | |
| Concentration of heparin (mgm. per 1 cc. of plasma).. | 0.00 | 0.08 | 0.16 | 0.24 | 0.32 | 0.40 |
| Thrombin (½ S.). Clotting time, seconds..... | 6 | 8 | 10 | 50 | 150 | No clot |
| Rabbit plasma dialyzed against distilled water: | | | | | | |
| Concentration of heparin (mgm. per 1 cc. of plasma).. | 0.00 | 0.40 | 0.80 | 1.20 | 1.60 | 2.00 |
| Thrombin (½ S.). Clotting time, seconds..... | 6 | 21 | 48 | 70 | 80 | 90 |
| Thrombin (F. S.). Clotting time, seconds..... | 4 | 7 | 9 | 10 | 12 | 13 |
| Dialyzed plasma with sodium chloride restored:* | | | | | | |
| Concentration of heparin (mgm. per 1 cc. of plasma).. | 0.00 | 0.40 | 0.80 | 1.20 | 1.60 | 2.00 |
| Thrombin (F. S.)..... | 5 | 30 | 240 | 480 | No clot | |

* Sufficient sodium chloride was added to approximate a concentration of 0.85 per cent.

Plasma was dialyzed for 2 hours in a Simms dialyzer, and 2 liters of distilled water were passed through the apparatus.

heparin is vastly more effective as an anticoagulant in plasma than in a solution of purified fibrinogen supports the contention of Howell that heparin does not directly antagonize thrombin, but produces in plasma a true antithrombin by reacting with an unknown constituent present in the blood. It is very likely that the slight anticlotting action of heparin observed in fibrinogen solutions is due to this antithrombin precursor carried down in the precipitation of fibrinogen from plasma, especially when ammonium sulfate is used. Since Mellanby employed a preparation of fibrinogen directly precipitated from plasma by ammonium sulfate, it

is quite probable that his product contained sufficient amounts of this antithrombin precursor to explain his results. It should be emphasized that Howell and his students isolated their preparation of fibrinogen from plasma by means of sodium chloride, and then purified it by repeated precipitations. Mellanby's contention that heparin is an antithrombin but only in the presence of neutral salts, and his implication that Howell's failure to observe the antithrombic action of heparin in solutions of purified fibrinogen was due to the absence of neutral salts, cannot be accepted. All of the present studies were carried out with fibrinogen solutions containing approximately 0.85 per cent sodium chloride.

TABLE 5

*The antithrombic potency of heparin when tested on fibrinogen* solutions prepared by different methods*

| Fibrinogen precipitated from plasma with ammonium sulfate | | | |
|---|---|---|---|
| Fibrinogen, cc | 0.2 | Fibrinogen, cc | 0.2 |
| Heparin, mgm | 0.2 | Thrombin (F. S.) | 0.1 |
| Thrombin (F. S.) | 0.1 | | |
| Clotting time, seconds | 17 | Clotting time, seconds | 45 |
| **Fibrinogen precipitated from plasma with sodium chloride** | | | |
| Fibrinogen, cc | 0.2 | Fibrinogen, cc | 0.2 |
| Heparin, mgm | 0.2 | Thrombin (F. S.) | 0.1 |
| Thrombin (F. S.) | 0.1 | | |
| Clotting time, seconds | 55 | Clotting time, seconds | 5 |
| Thrombin ($\frac{1}{20}$ S.), cc | 0.1 | Thrombin ($\frac{1}{20}$ S.), cc | 0.1 |
| Clotting time, seconds | 34 | Clotting time, seconds | 20 |

* Fibrinogen obtained from human plasma.

The action of heparin on purified fibrinogen dissolved in distilled water was interesting, for invariably a precipitate was formed. Likewise on dialyzing plasma containing heparin against distilled water, a flocculation appeared. It is difficult to known whether a direct union of heparin with fibrinogen takes place forming a complex which is insoluble in distilled water; nevertheless, the interesting possibility arises that heparin may perhaps possess the property of combining with plasma proteins.

*3. The anticoagulative action of heparin is apparently not influenced by thromboplastin.* In attempting to determine whether thromboplastin per se can neutralize heparin, it is extremely important to eliminate all traces of prothrombin. In the preceding experiments it has been shown

that the inhibitory action of heparin can be overcome by increasing the quantity of thrombin. Obviously, therefore, if any reagents are present which contain traces of prothrombin, additional thrombin will form in the presence of calcium and thromboplastin, and this will counteract the anticlotting action of heparin. In failing to recognize this possibility, one can easily be led into the error of ascribing the observed acceleration of clotting to a direct neutralization of heparin by thromboplastin rather than to the formation of additional thrombin. The simplest means for studying the action of thromboplastin on heparin consists in mixing fixed amounts of purified fibrinogen, heparin, and thromboplastin, and then adding thrombin. By comparing the clotting time of such a system

TABLE 6

*The inability of thromboplastin to nullify the inhibitory action which heparin exerts in the clotting of fibrinogen* by thrombin*

| | | | |
|---|---|---|---|
| Fibrinogen, cc. | 0.2 | Fibrinogen, cc. | 0.2 |
| Heparin, mgm. | 0.2 | Heparin, mgm. | 0.2 |
| Thromboplastin, cc. | 0.1 | Normal saline, cc. | 0.1 |
| Thrombin ($\frac{1}{5}$ S.), cc. | 0.1 | Thrombin ($\frac{1}{5}$ S.), cc. | 0.1 |
| Clotting time, seconds | 23 | Clotting time, seconds | 23 |
| Fibrinogen, cc. | 0.2 | Fibrinogen, cc. | 0.2 |
| Heparin, mgm. | 0.4 | Heparin, mgm. | 0.4 |
| Thromboplastin, cc. | 0.1 | Normal saline, cc. | 0.1 |
| Thrombin ($\frac{1}{5}$ S.), cc. | 0.1 | Thrombin ($\frac{1}{5}$ S.), cc. | 0.1 |
| Clotting time, seconds | 43 | Clotting time, seconds | 41 |

* Fibrinogen was prepared from human plasma with ammonium sulfate, treated with aluminum hydroxide to remove prothrombin, and dissolved in normal saline solution.

with a control in which physiological saline solution is substituted for thromboplastin, useful data concerning the effect of thromboplastin on heparin can be obtained. The results recorded in table 6 definitely suggest that thromboplastin does not antagonize the inhibitory action which heparin has on the coagulation of fibrinogen by thrombin. Interestingly, when impure preparations of fibrinogen, ones containing traces of prothrombin, are used, thromboplastin will be found to have a moderate accelerative action on the clotting time which has been delayed by heparin, as shown in table 7. It is quite certain that the additional thrombin, derived from the prothrombin present as an impurity, is responsible for counteracting the anticlotting action of heparin, since this action of thromboplastin is not observed when prothrombin-free fibrinogen is em-

ployed. This pseudo-neutralization demonstrates how easily one can be led to misleading and erroneous conclusions by impure reagents.

In view of the relatively weak inhibitory action that heparin has on the clotting of purified fibrinogen by thrombin, objections may justifiably be raised against using such an agent for investigating the action of thromboplastin on heparin. Fortunately another means for this study was found which appears to be exceptionally satisfactory. In this as well as in former papers, the author has pointed out the remarkable property of aluminum hydroxide to remove prothrombin from plasma without apparently causing any other demonstrable change. No evidence has been found that fibrinogen suffers any physical or chemical change; it seems certain that the concentration of electrolytes is only slightly altered since the calcium can be restored; and heparin added to this plasma still forms an antithrombin. Significantly, thrombin converts the fibrinogen in alumina plasma as readily to fibrin as in ordinary oxalated plasma.

TABLE 7

*Pseudo-neutralization of heparin by thromboplastin when prothrombin is present as an impurity*

| | | | |
|---|---|---|---|
| Fibrinogen,* cc. | 0.2 | Fibrinogen,* cc. | 0.2 |
| Heparin, mgm. | 0.4 | Heparin, mgm. | 0.4 |
| Thromboplastin, cc. | 0.1 | Normal saline, cc. | 0.1 |
| Thrombin (⅛ S.), cc. | 0.1 | Thrombin (⅛ S.), cc. | 0.1 |
| Clotting time, seconds | 21 | Clotting time, seconds | 38 |

* Fibrinogen solution prepared by direct precipitation from plasma with ammonium sulfate. No treatment with aluminum hydroxide. On recalcification, it clotted within an hour, thus showing the presence of prothrombin.

In employing this prothrombin-free plasma for testing the action of thromboplastin on heparin, a constant quantity of alumina plasma was mixed with an optimal amount of calcium chloride, a fixed excess of thromboplastin, and increasing amounts of heparin. The clotting times of this series were determined after the addition of a fixed quantity of thrombin. As a control the clotting times of a similar series were observed in which an equal volume of 0.85 per cent sodium chloride solution was substituted for the thromboplastin emulsion. Strikingly, no difference was found between the mixtures containing thromboplastin and those which did not contain this agent (see table 8), demonstrating that the progressive retarding influence of increasing amounts of heparin on the clotting time is not antagonized by thromboplastin. It is difficult to offer any explanation other than that thromboplastin is completely inert and ineffective against the anticoagulative action of heparin provided prothrombin is absent.

Interestingly, thrombin, which is remarkably stable in pure solution as demonstrated by the fact that preparations made by the method of Eagle can be kept over a week without any appreciable loss of activity, nevertheless disappears rapidly in serum. On adding a concentrated thrombin solution to serum, it likewise disappears suggesting the presence of a great excess of antithrombin in serum. On adding thromboplastin to this serum which has neutralized the added thrombin, no thrombin is regenerated or liberated as seen by the experiments recorded in table 9. These findings are fully in accord with the older studies of Dale and Walpole (18) and of Gasser (19). These investigators likewise were unable to demonstrate that thromboplastin could neutralize antithrombin or liberate thrombin from metathrombin. While one does not know what relation the antithrombin of serum has to the antithrombic substance produced by

TABLE 8

*The inability of thromboplastin to counteract the inhibitory influence which heparin exerts on thrombin in prothrombin-free plasma*

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Heparin, mgm. | 0.00 | 0.01 | 0.02 | 0.03 | 0.04 | 0.05 | 0.06 | 0.07 | 0.10 |
| Prothrombin-free plasma,* cc. | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Calcium chloride 0.025 M., cc. | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Thromboplastin, cc. | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Thrombin (F. S.), cc. | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Clotting time, seconds | 4 | 18 | 32 | 60 | 80 | 120 | 210 | 330 | No clot |
| Clotting time of control,† seconds | 4 | 13 | 26 | 50 | 81 | 120 | 225 | 330 | No clot |

* Human plasma treated with aluminum hydroxide.

† Normal saline solution substituted for thromboplastin.

heparin, it is interesting and perhaps significant that neither one is neutralized by thromboplastin.

*4. The effectiveness of heparin in inhibiting the acceleration of clotting by thromboplastin is much greater in human than in rabbit plasma.* By adding increasing amounts of heparin to oxalated human plasma, a concentration is eventually reached which will completely inhibit clotting even though an excess of thromboplastin and an optimal amount of calcium chloride are present. Much larger quantities added to rabbit plasma exert, in marked contrast, relatively little inhibitory action on clotting when excess thromboplastin is supplied (table 10). A simple explanation for this difference can be offered. The author (20) by means of his method for determining prothrombin has found that human plasma contains much less of this clotting factor than does rabbit plasma, and therefore can

yield less thrombin. From the results of the preceding experiments, as well as from the work of Howell and his co-workers, evidence was obtained that heparin added to plasma gives rise to a strong antithrombin. Thus, it can be assumed that when heparin is added either to rabbit or human plasma, a definite quantity of antithrombin is formed, but the amount of

TABLE 9

*The inability of thromboplastin to neutralize the antithrombin of serum or to bring about regeneration of thrombin from metathrombin*

| | | | |
|---|---|---|---|
| Fibrinogen, cc. | 0.1 | Fibrinogen, cc. | 0.1 |
| Thromboplastin, cc. | 0.1 | Normal saline, cc. | 0.1 |
| Serum-thrombin mixture,* cc. | 0.1 | Serum-thrombin mixture, cc. | 0.1 |
| Clotting time, seconds | 40 | Clotting time, seconds | 40 |

* The serum-thrombin mixture consisted of 1 cc. thrombin (F. S.) and 1 cc. of rabbit serum which was 9 hours old. The mixture was allowed to stand 5 minutes before it was used. After 15 minutes it had nearly completely lost its thrombic activity. Fibrinogen prepared from rabbit plasma was used.

TABLE 10

*The difference in the effectiveness with which thromboplastin accelerates the coagulation of recalcified human and rabbit plasma to which heparin has been added*

| Concentration of heparin (mgm. per 1 cc. of plasma) | 0.00 | 0.05 | 0.10 | 0.15 | 0.20 | 0.25 | 0.30 | 0.40 | 0.50 |
|---|---|---|---|---|---|---|---|---|---|
| Clotting time* of human plasma, seconds | 16 | 16 | 18 | 21 | 22 | 31 | 40 | 60 | No clot |
| Clotting time after 1 hour incubation, seconds | 16 | 17 | 20 | 24 | 26 | 40 | 58 | 450 | No clot |

| Concentration of heparin (mgm. per 1 cc. of plasma) | 0.00 | 0.20 | 0.40 | 0.60 | 0.80 | 1.00 | 1.20 | 1.60 | 2.00 |
|---|---|---|---|---|---|---|---|---|---|
| Clotting time of rabbit plasma, seconds | 8 | 9 | 11 | 12 | 16 | 18 | 25 | 30 | 35 |

* 0.1 cc. of plasma was mixed with 0.1 cc. of thromboplastin, and the clotting time determined after the addition of 0.1 cc. 0.025 M. calcium chloride.

thrombin formed when excess thromboplastin and an optimal amount of calcium are added is much greater in rabbit than in human plasma. Therefore the amount of heparin which is adequate for the production of enough antithrombin to neutralize all the thrombin that human plasma can yield is far short of the amount required to inactivate the total throm-

bin formed in rabbit plasma. Strong experimental support for this explanation is found in table 11. By diluting rabbit plasma with prothrombin-free plasma (plasma treated with aluminum hydroxide) until the prothrombin content as measured by the clotting time obtained with an excess of thromboplastin and an optimal amount of calcium is the same as that of human plasma, a mixture should be obtained which exhibits the same response to heparin as normal human plasma. Significantly rabbit plasma thus diluted fulfills the postulated requirements.

On the well founded assumption that heparin gives rise to an antithrombin, it can easily be understood how thromboplastin by accelerating the speed of the conversion of prothrombin to thrombin can antagonize the action of heparin. But it must be pointed out that the effectiveness of thromboplastin is strictly limited by the available quantity of prothrombin and is not dependent upon a direct neutralization of heparin. Con-

TABLE 11

*The action of thromboplastin on a plasma having approximately the same prothrombin concentration as human plasma, prepared by diluting rabbit plasma with prothrombin-free plasma*

| Concentration of heparin (mgm. per 1 cc. of plasma) | 0.00 | 0.30 | 0.40 | 0.50 |
|---|---|---|---|---|
| Clotting time† of synthetic plasma,* seconds. | 18 | 50 | 65 | 140 |
| Clotting time of human plasma, seconds. . . | 18 | 50 | 67 | 150 |

* Synthetic plasma was prepared by mixing 1.3 cc. of oxalated rabbit plasma with 4.5 cc. of human plasma that had been treated with aluminum hydroxide.

† 0.1 cc. of plasma was mixed with 0.1 cc. of thromboplastin, and the clotting time determined after the addition of 0.1 cc. 0.025 M. calcium chloride.

sequently, if the amount of heparin or rather the antithrombin it produces exceeds the quantity of thrombin that the plasma can yield, no coagulation can occur no matter how much excess thromboplastin is supplied. If thromboplastin neutralized heparin directly, it would be logical to expect that a fixed excess of tissue extract added to either human or rabbit plasma containing the same amount of heparin, should be equally potent in either plasma instead of exhibiting the marked difference experimentally observed.

It will be observed that heparin becomes effective immediately after it is added to plasma, but nevertheless its anticlotting potency increases slightly on standing and as the concentration of heparin approaches the critical level at which the action of thrombin is completely inhibited, the increase is sufficient to exert a rather marked effect. By selecting a proper concentration of heparin and a preparation of thromboplastin of known

strength, and using normal human plasma, which the author has found to be remarkably constant in regard to its clotting factors, one can titrate heparin employing the formation of a clot, or better the failure to clot as the end point.

COMMENT. In agreement with Howell, the present results support the view that heparin per se cannot neutralize thrombin but that it produces a strong antithrombin when added to plasma. The results of Mellanby are at variance with this hypothesis, but as has already been pointed out, the fibrinogen which he employed probably contained sufficient impurities to invalidate his conclusions. Nevertheless his work contributes a new important fact, namely, the necessity of neutral salts for the activity of heparin.

Contrary to the widely accepted theory that thromboplastin neutralizes heparin directly, the observations presented in the present paper suggest that the actions of heparin and of thromboplastin are entirely independent of each other; the former giving rise to a strong antithrombin, the latter producing thrombin by reacting with prothrombin. It can readily be seen, however, that the action of thromboplastin may simulate the neutralization of heparin, since it can by its ability to convert any traces of prothrombin (accidentally present as an impurity in fibrinogen or any other agent) to thrombin and thus accelerate clotting. But obviously this effect is not due to the inactivation of heparin but to the additional thrombin.

Convincing evidence was obtained from the study of the direct action of thrombin on plasma than no appreciable amounts of free heparin are present in the latter medium, in fact the linear relationship found between the clotting time of plasma and the concentration of thrombin fairly definitely precludes the existence of any antithrombin. This is in marked contrast to the large amount of antithrombin in serum, which is sufficient not only to inactivate the thrombin produced from the prothrombin originally present but also to neutralize large amounts of added thrombin.

The abundance of antithrombin in serum and the absence of it in plasma leads to the rather surprising possibility that the antithrombin may perhaps form during the actual process of coagulation. There is a possibility that heparin may furnish the key to this problem. Unfortunately so little is definitely known concerning its action that one must still resort to speculation. If heparin is not itself an antithrombin, how does it produce this agent in plasma? Perhaps the correlation of certain observations may be helpful. In recent years Fischer (21) has demonstrated that heparin appears to have an affinity for serum proteins and that it can actually effect a marked change in their properties, such as shifting their isoelectric point. Rettger (15) and Landsberg (22) from their studies of antithrombin came to the conclusion that the thrombin-neutralizing substance might be a plasma protein. This supposition is supported by the

fact that both serum proteins and antithrombin exhibit roughly the same lability to heat. The two concepts, that of Fischer, and that of Rettger and Landsberg, hitherto unrelated, lead to an interesting speculation. May not perhaps heparin unite with a plasma protein and thereby confer on it the property of combining with thrombin and so convert it into an antithrombin, and that the normally occurring antithrombin of serum may arise from the liberation of a heparin-like substance?

## SUMMARY

1. The relation between the clotting time and the concentration of thrombin can be expressed by the simple linear equation:

$$\text{Clotting time} = k \frac{1}{\text{concentration of thrombin}}$$

The corrective factor, $k$, is dependent upon the concentration of electrolytes. It decreases with increasing concentrations of neutral salts. The direct ratio between the speed of clotting and the quantity of thrombin is strong evidence against the presence of free heparin in plasma, since the addition of even minute amounts to plasma destroys this linear relationship.

2. Heparin is not an antithrombin since it possesses only a slight inhibitory action on thrombin when tested with purified fibrinogen, in strong contrast to the strong anticlotting action it produces in plasma. Heparin appears to react with a constituent present in plasma to form a true antithrombin. In this reaction neutral salts are necessary. In plasma in which electrolytes are removed by dialysis against distilled water, heparin is inactive.

3. Evidence was obtained indicating that thromboplastin does not directly neutralize heparin:

A. The clotting of prothrombin-free plasma by thrombin is progressively delayed by increasing concentrations of heparin. This inhibitory effect of heparin is not abolished or even influenced by excess thromboplastin and an optimal quantity of calcium.

B. The acceleration in clotting time of oxalated human plasma by excess thromboplastin and an optimal amount of calcium is delayed and ultimately completely inhibited by increasing amounts of heparin. In rabbit plasma, which contains a higher concentration of prothrombin, heparin is comparatively less effective in antagonizing the action of thromboplastin.

The present observations are most satisfactorily explained by the assumption that thromboplastin antagonizes the anticlotting action of heparin by accelerating the conversion of prothrombin to thrombin and

that the latter is the true antagonist of the inhibitory substance heparin produces in plasma.

## REFERENCES

(1) Howell, W. H. and E. Holt. This Journal **47**: 328, 1918.
(2) Howell, W. H. This Journal **71**: 553, 1924.
(3) Howell, W. H. Bull. Johns Hopkins Hosp. **42**: 199, 1928.
(4) Mellanby, J. Proc. Roy. Soc. B. **107**: 271, 1930.
(5) Schmitz, A. and L. Kuhl. Ztschr. physiol. Chem. **234**: 212, 1935.
(6) Waldschmidt-Leitz, E., P. Stadler and F. Steigerwaldt. Ztschr. physiol. Chem. **183**: 39, 1929.
(7) Howell, W. H. Physiol. Rev. **15**: 435, 1935.
(8) Eagle, H. J. Gen. Physiol. **18**: 531, 1935.
(9) Quick, A. J. This Journal **114**: 282, 1936.
(10) Simms, H. S. J. Exper. Med. **51**: 319, 1930.
(11) Scott, D. A. and A. F. Charles. J. Biol. Chem. **102**: 437, 1933.
(12) Schmitz, A. and A. Fischer. Ztschr. physiol. Chem. **216**: 264, 1933.
(13) Jorpes, E. Die Naturwissenschaften **23**: 196, 1935; Biochemical J. **29**: 1817, 1935.
(14) Ducleaux, E. and M. Arthus. Quoted by E. Wohlisch. Ergebn. d. Physiol. **28**: 443, 1929.
(15) Rettger, L. J. This Journal **24**: 406, 1909.
(16) Martin, C. J. J. Physiol. **32**: 201, 1905.
(17) Barratt, J. O. W. J. Physiol. **80**: 423, 1934.
(18) Dale, H. H. and G. St. Walpole. Biochem. J. **10**: 331, 1916.
(19) Gasser, H. S. This Journal **42**: 378, 1917.
(20) Quick, A. J. J. Biol. Chem. Proc. **109**: lxxiii, 1935.
(21) Fischer, A. Biochem. Ztschr. **244**: 464, 1932.
(22) Landsberg, M. Biochem. Ztschr. **50**: 245, 1913.

# CHANGES IN THE RAT INCISOR FOLLOWING BILATERAL ADRENALECTOMY[1]

I. SCHOUR AND J. M. ROGOFF

*From the Department of Histology, College of Dentistry, University of Illinois and the Physiological Laboratory, University of Chicago*

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Sensitive responses of the rat incisor to parathyroidectomy (Erdheim, 1906) and hypophysectomy (Schour and Van Dyke, 1932) and of the incisor of the ground squirrel to bilateral gonadectomy (Schour, unpublished) have been observed. This has suggested the advisability of investigating the possible effect of adrenalectomy, which to our knowledge has not been studied hitherto.

Forty-five young rats, ranging in weight from twenty-seven to ninety-five grams, were obtained from the colony of the Physiological Laboratory, University of Chicago. A number of animals from the same stock were available as controls in addition to a considerable number that had been used as controls in the course of other investigations. All the experimental animals were kept in individual cages and under similar conditions, on the "Sherman B" diet. Bilateral adrenalectomy, post-operative care and autopsies were performed by one of us (J. M. R.) in his laboratory. Careful search was made for accessory adrenal bodies and suspected tissues were preserved for microscopic examination. Each animal was identified by number. The heads were removed as soon as possible after death, preserved in formalin and delivered to the laboratory of the other co-worker (I. S.) for histological preparation and examination, in which valuable assistance was rendered by M. M. Hoffman. Neither collaborator had any knowledge of the records on the experiments made by the other until completion of the work, when the notes made by both were exchanged for proper analysis of the results.

Of the forty-five rats that were subjected to bilateral adrenalectomy, eight died within one to three days. In these, death was due not only to loss of the adrenals but to contributing causes associated with the operation. Thirty rats died of uncomplicated acute adrenal insufficiency, within the usual period of survival obtained in our experience, twelve on the fourth day, six on the fifth, one on the sixth, three on the seventh, one

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on the ninth, two on the thirteenth, two on the fourteenth, two on the fifteenth and one on the twentieth day (table 1). The remaining seven animals were sacrificed, three on the forty-third and four on the forty-fourth day after adrenalectomy. Hypertrophied accessory adrenal bodies were found in six of these and in the seventh careful macroscopic examination failed to reveal any accessory tissue. Characteristic changes were found in the incisors of all except one (no. 12) of the animals that survived

TABLE 1

*Survival of adrenalectomized rats compared with survival period calculated from zoning in dentin*

| NUMBER OF ANIMAL | SEX | WEIGHT | SURVIVAL | | NUMBER OF ANIMAL | SEX | WEIGHT | SURVIVAL | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Recorded | Calculated | | | | Recorded | Calculated |
| | | *grams* | *days* | *days* | | | *grams* | *days* | *days* |
| 3 | M. | 56.5 | 1− | 0.97 | 34 | M. | 43.5 | 4 | 3.8 |
| 28 | M. | 30 | 1 | 0.87 | 9 | M. | 76 | 5− | 5.3 |
| 30 | M. | 27 | 1 | 1.09 | 25 | M. | 36.5 | 5− | 4.8 |
| 36 | M. | 34 | 1 | 1.2 | 33 | M. | 44 | 5− | 4.8 |
| 38 | M. | 34 | 2 | 2.0 | 40 | M. | 31.5 | 5− | 4.9 |
| 44 | M. | 33 | 2 | 2.1 | 41 | M. | 33 | 5 | 5.1 |
| 27 | M. | 30 | 3− | 3.0 | 43 | M. | 58 | 5 | 5 |
| 35 | M. | 33 | 3− | 2.8 | 42 | M. | 51 | 6 | 6 |
| 4 | M. | 59.5 | 4− | 3.9 | 18 | F. | 60 | 7− | 6.7 |
| 12 | M. | 68 | 4− | 3.9 | 45 | M. | 43.5 | 7− | 6.6 |
| 20 | M. | 66.5 | 4− | 4.1 | 10 | F. | 58.5 | 7 | 7.4 |
| 23 | M. | 32 | 4− | 3.4 | 11 | M. | 69.5 | 9− | 9 |
| 26 | M. | 33 | 4− | 4.1 | 8 | M. | 60.5 | 13− | 12.9 |
| 29 | M. | 30.5 | 4− | 3.8 | 14 | M. | 86 | 13 | 13.2 |
| 31 | M. | 32 | 4− | 3.8 | 1 | F. | 86 | 14− | 13.3 |
| 37 | M. | 35 | 4− | 3.9 | 5 | M. | 94 | 14− | 13.3 |
| 15 | M. | 95.5 | 4 | 3.9 | 13 | F. | 93 | 15 | 14.8 |
| 16 | F. | 83.5 | 4 | 3.9 | 21 | F. | 66 | 15 | 15.4 |
| 19 | F. | 56.5 | 4 | 4.1 | 2 | M. | 87.5 | 20− | 20.3 |

The minus indicates that the animal died during the preceding night.

up to twenty days, including the eight that survived only one to three days and died of other causes complicating adrenal insufficiency. The experimental reaction was found in the upper incisors more readily than in the lower. In rat 12, the reaction was doubtful. In the seven rats that were sacrificed later, the teeth showed changes similar to those associated with rickets.

A histologic study was made of one or two incisors of each animal. The teeth with their investing tissues, fixed in formalin, were examined and photographed under x-ray. Then they were washed, decalcified in

5 per cent nitric acid, embedded in celloidin and stained with hematoxylin and eosin. The sections were cut longitudinally and mounted in serial order. Tangential sections of the predentin should be avoided or interpreted with special caution, since they may have an appearance simulating globular predentin, even in the normal condition, and may lead to error. Micrometer readings were made of the zoning and stratification in the dentin.

For comparison, the histology of dentin in the normal rat incisor will be presented briefly, since the characteristic changes observed in our experimental animals occurred in the earlier calcification processes of dentin. The dentin is a very highly specialized connective tissue. Its organic matrix is laid down along the surface of the dental pulp, at the rate of 16 $\mu$ per twenty-four hours (Schour and Smith, 1934). Calcification of the matrix normally proceeds in close chronological succession with its formation.

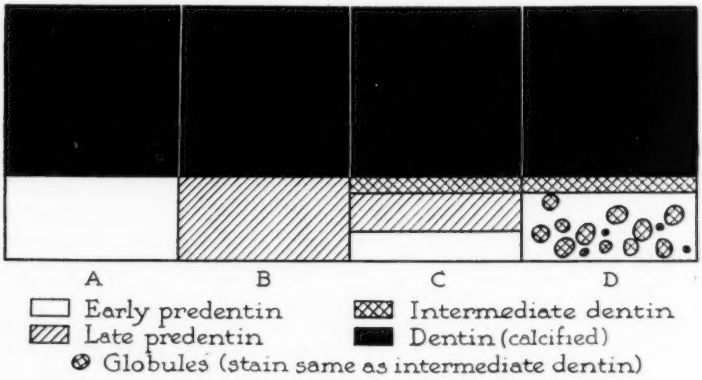


Fig. 1. Schematic description of staining reactions in predentin

In unoperated control rats the predentin usually takes the eosin color when stained with hematoxylin and eosin. The different staining reactions of predentin in the incisor of the normal rat and of the adrenalectomized rat are illustrated schematically in figure 1. Normally, the predentin presents one of the following three staining reactions. 1. Sometimes the entire width of the predentin may be non-staining (fig. 1, A). This is referred to as "early" predentin (Schour, Tweedy and McJunkin, 1934). 2. The entire width of the predentin may stain with eosin (fig. 1, B). This may be considered as "late" predentin (eosinophilic). 3. Occasionally, the portion next to the pulp does not stain (early predentin), the middle portion staining with eosin (late predentin), and that adjacent to the calcified dentin taking both the eosin and hematoxylin stains (fig. 1, C). The latter portion is referred to as intermediate dentin. In the predentin of adrenalectomized rats there is an interspersion of globules (globular predentin) which stain with both eosin and hematoxylin (fig. 1, D).

Calcification of the dentin matrix occurs in the form of globules which increase

in number and size and coalesce until the dentin is homogeneously calcified. In decalcified sections the dentin stains predominantly with hematoxylin (basophilic). When calcification is not complete a varying proportion of uncalcified matrix remains within the calcified globular dentin in the form of eosin-staining interglobular dentin.

*Changes in the incisor of the rat following adrenalectomy. a. Predentin.* In the predentin of the incisors of adrenalectomized rats are found disseminated globules that vary in size and number and take an intermediate stain of hematoxylin and eosin. These are not found in unoperated control rats, the predentin either staining homogeneously with eosin or not staining at all. The globules occur within the early and late predentin and those close to the pulp are usually smaller than those nearer the dentin. To our knowledge this type of predentin has not been reported in the literature previously and, for convenience, we shall refer to it as *globular predentin.* Its presence in the incisors of rats that have been subjected to bilateral adrenalectomy is illustrated in figure 2 (no. 1 and 2). The globular predentin is distinct. Within the eosin-staining late predentin, *L*, are seen the globules, *Gl*, which stain like the intermediate dentin, *In*, with little or no predominance of either eosin or hematoxylin. In figure 2, no. 1 is from a rat that survived nearly 9 days (table 1, animal 11) and 2 from an animal surviving less than twenty-four hours (table 1, animal 3). The fields were taken from longitudinal sections of the upper incisors, at the level of the distal end of the organic enamel matrix, where globular predentin is found when present. The older dentin (D) is well calcified.

Globular predentin appears first at the level of the tooth where the organic enamel matrix has reached its full width and it extends through the proximal third of the enamel space. It is absent in approximately the distal third of the incisor (fig. 2, no. 5, 6, 7). Globular predentin, as observed by us in rats which died of adrenal insufficiency within about four weeks, has not been found in histological sections of incisors in any of nearly one thousand rats and their controls studied in previous investigations by one of us (I. S.), except in three animals that had received large doses of parathormone. These animals will be referred to later. In the seven animals that were sacrificed (43 and 44 days after adrenalectomy) and in which long survival was unquestionably sustained by presence of accessory adrenal bodies, the predentin was not globular but was abnormally wide (40–84 $\mu$). These animals presented changes similar to those found in rickets.

*b. Dentin.* The dentin that was formed and calcified during the period of survival after adrenalectomy differed from that of the pre-operative period. It will be seen in figure 2 (no. 5) that the post-operative dentin, $D_2$, is stained much more deeply than the pre-operative, *D*. The dentin

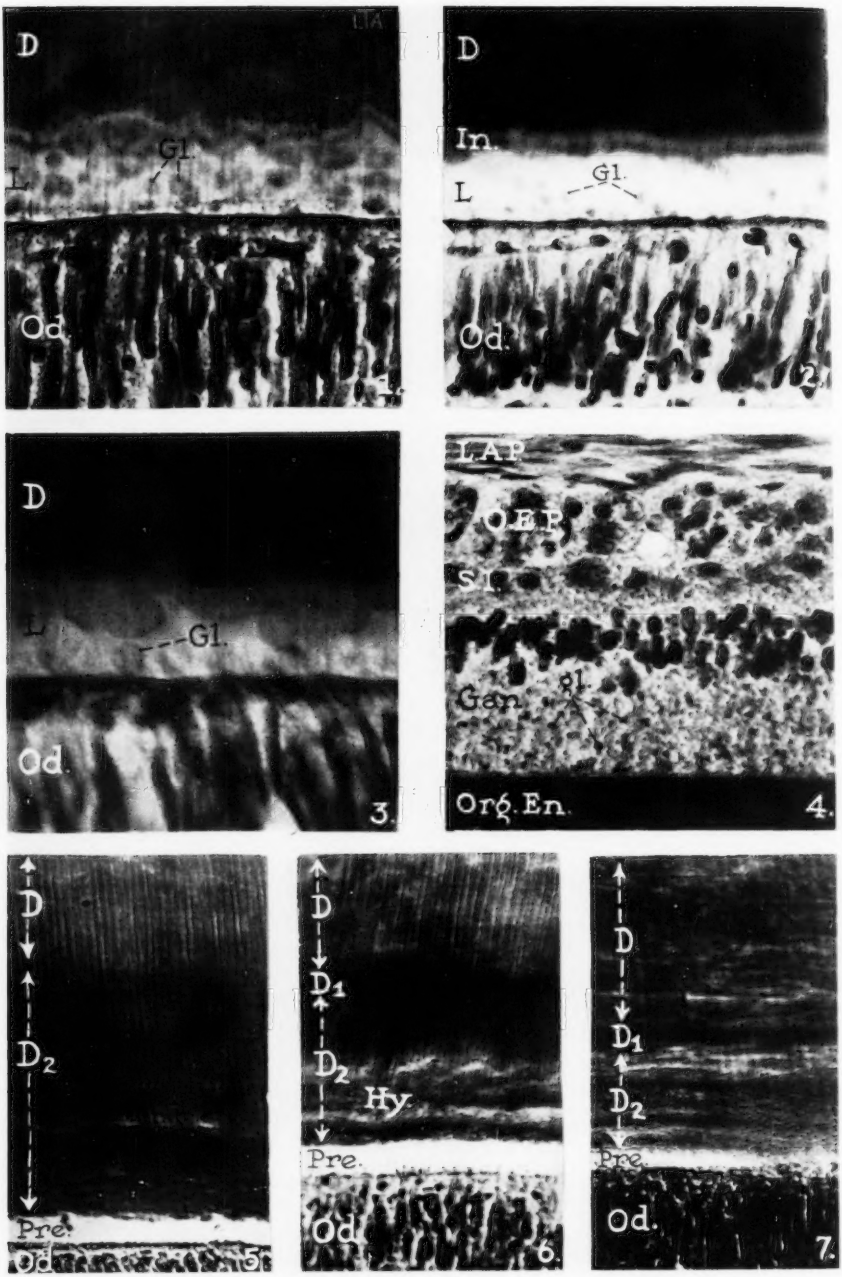


Fig. 2. Photomicrographs of longitudinal sections of rats' incisors. 1, 2, ×490; 3, × 610; 4, × 560; 5, × 220; 6, 7, × 280.

that was formed and calcified at the time of the operation usually presents a distinct deep hematoxylin stripe (fig. 2, no. 6, 7; $D_1$). This was more commonly found in the teeth of adrenalectomized rats that survived less than ten days. In some cases the entire post-operative dentin, $D_2$, stained deeply with hematoxylin (no. 5), in others the post-operative dentin showed stratification that was more prominent than normal (no. 6, 7).

The identity of the post-operative dentin (from its deep staining with hematoxylin, or in cases where the hematoxylin stripe distinguished it from the pre-operative) made it possible to measure its width. Since the organic matrix of the dentin is laid down at the rate of 16 $\mu$ per day, we were able to calculate the period of survival by dividing the width of the post-operative dentin, in $\mu$, by 16. It is surprising how closely the calculated survival period corresponded with the actual survival period, especially in those animals that lived up to about ten days (table 1). Beyond this period there are often found additional deeply stained stripes that may be confusing. However, if a number of measurements are made, the distinguishing stripe usually will be found and will yield the calculated survival period closely corresponding to the actual. These are the figures that are included in table 1 for the last seven animals, surviving thirteen to twenty days. They showed a stratification or "zoning" in the post-operative dentin which included an additional stripe that coincided in position with the time of operation.

Figure 2, no. 5, is a photomicrograph of a longitudinal section in the mid-region of an upper incisor of rat no. 1. In this field the predentin, *Pre*, is chiefly of the early type and is seen adjacent to the odontoblasts, *Od*. The dentin that was formed and calcified before the operation, *D*, is less deeply stained than the post-operative dentin. The deeply stained post-operative dentin, $D_2$, measured 213 $\mu$ in width. Dividing by 16 gives the calculated survival period as 13.3 days. The experimental record shows that the rat survived between thirteen and fourteen days (table 1). In rat 18 (fig. 2, no. 6) a photomicrograph of a longitudinal section of the labial dentin, *D*, of the upper incisor, taken at the level of the alveolar crest, shows the deeply stained band, $D_1$, which marks the level of dentin formed and calcified at the time of operation for adrenalectomy. The band is 107 $\mu$ from the pulp. Dividing this by 16 gives 6.7 days as the calculated survival period. The record shows that the animal survived between six and seven days (table 1). Within the post-operative dentin, $D_2$, are seen hypocalcified bands, *Hy*, the significance of which is unknown. The suggestion arises that they possibly might be associated with those metabolic disturbances which, when exaggerated, lead to the tetanic convulsions that commonly occur in adrenalectomized animals. The early predentin, *Pre*, is clearly seen in this field.

In photomicrograph 7 (fig. 2) is shown a longitudinal section of the

lingual dentin, $D$, of an upper incisor of adrenalectomized rat no. 25. The band, $D_1$, which marks the level of dentin laid down and calcified at the time of the operation, is located 78 $\mu$ from the pulp, which gives a calculated survival period of 4.8 days. The recorded survival was between 4 and 5 days (table 1). Disturbances in calcification are indicated by the presence of hyper- and hypocalcified bands within the post-operative dentin, $D_2$.

*Zoning in dentin.* Analysis of our measurements of the dentin shows a high incidence of zoning which can be correlated with the experimental history of the animals and particularly with the time of operation. The deeply stained stripe that indicates the dentin laid down and calcified at the time of operation was the only prominent band present in the labial dentin in each of the animals that survived under nine days and was present, together with other stripes, in those animals surviving nine to twenty days. It was not present in the seven animals that were permitted to survive 43 and 44 days. In these, rachitic-like changes were present in the dentin in the form of prominent interglobular dentin and vascular inclusions. The predentin was abnormal in width and was not globular. The absence of the prominent band in these animals can be explained by the fact that the dentin laid down at the time of operation had moved so distant during the long period of survival that it may have disappeared entirely. Also, it may be difficult to recognize because of its distant position from the pulp or it may be obliterated through secondary calcification. Histologic study of comparable zones of dentin in the labial and lingual portions of the incisor indicates a possible difference in degree of their sensitivity to the same systemic disturbance. Further study of this phenomenon may yield information of interest on the biology of the tooth.

*c. Enamel-forming cells (Ganoblasts).* In some of the experimental animals, the ganoblasts in the proximal third of the incisor, especially at the level where the epithelial papillae begin to appear larger, contain distinct hematoxylin-staining globules within the cytoplasm. In some cells they are confined to the distal end, in others they are scattered throughout the cytoplasm and give the latter an appearance that might be described as resembling a *hailstorm.* These globules vary in size from approximately 1 $\mu$ to 4 $\mu$. They must be carefully distinguished from artifacts sometimes found in this area as a result of postmortem changes. The globules are illustrated in figure 2 in a photomicrograph (no. 4) of a longitudinal section of an upper incisor of rat 34 which survived four days after bilateral adrenalectomy. The field is at the level of the mid-region of the organic enamel matrix. This section shows the globules, *gl*, which stain with hematoxylin, scattered throughout the cytoplasm of the ganoblasts, *Gan.* The outer layer of the enamel epithelium arranged

in low epithelial papillae, *O. E. P.*, the stratum intermedium, *S. I.*, the organic enamel matrix, *Org. En.*, and the labial alveolar periosteum, *L. A. P.*, are shown.

DISCUSSION. These experiments indicate that the presence of globular predentin in the incisor is a characteristic phenomenon following bilateral adrenalectomy in the rat. Its occurrence only in those animals that survived up to twenty days suggests that it is associated with acute or subacute adrenal insufficiency. Animals that lived beyond the usual survival period of completely adrenalectomized rats were found to possess accessory adrenal bodies. They presented changes in the teeth similar to those seen in rickets.

The constancy with which globular predentin is found in adrenalectomized rats suggests the advisability of investigating the possibility of utilizing this reaction in testing or standardizing adrenal cortical extracts. However, it is not certain that the reaction is caused by cortical insufficiency alone. The present evidence cannot eliminate the medulla as a possible factor.

In the course of this investigation we were impressed with the importance of certain conditions that apparently determine consistent results. Other series of experiments yielded similar but not as unequivocal results as those reported here. Animals were obtained from various sources, some were much older than those used in the present series, some had been used previously for other experiments and other conditions prevailed, all of which were eliminated in this series.

Our experiments do not determine whether the changes found in the predentin and dentin of the incisor, in adrenalectomized rats, indicate deficient or excessive calcification. The presence of globular predentin suggests the probability of premature or excessive calcification. In guinea pigs, calcification of predentin is associated with vitamin C deficiency. However, the globular condition is absent (Schour, unpublished).

The hematoxylin-staining globules observed in the ganoblasts, in a number of the experimental animals (fig. 2, no. 4) may represent a counterpart of the globules found in the predentin (fig. 2, no. 1 and 2). Their presence suggests the probability of disturbance in formation and also in calcification of enamel. However, their significance cannot be determined without further investigation. At present, the evidence is not conclusive that the globular appearance within the ganoblasts is as constant a characteristic of adrenalectomy as the globular predentin.

The consistent observation of rachitic-like changes in the seven adrenalectomized rats that survived 43 or 44 days suggests a possible relationship between chronic adrenal insufficiency and rickets. This will be investigated further. Stoeltzner reported favorable results in the treatment of rickets with adrenal preparations but other investigators were

unable to confirm his observations (Goldblatt, 1931). It is not probable that the rachitic-like changes were due to inadequate diet since they were absent in all of the other 38 rats, kept on the same diet. The survival period of some of these rats (up to 20 days) was sufficiently long to permit development of rickets if the diet is rachitogenic (Becks and Ryder, 1934). It may be mentioned, also, that the seven rats had gained weight eleven days after adrenalectomy while the weight of the others declined or remained unchanged.

Similar results were observed in another series of experiments which, incidentally, illustrate the advisability of using animals from a source known to be suitable. Two litters of seven rats each, from different sources, had been used for controlling a study on the effect of various diets upon survival of adrenalectomized rats. The animals ranged from 42 grams to 80 grams body weight. Three different diets were employed. Regardless of diet, six animals of one litter died in 5, 6, 7, 8, 27 and 30 days respectively while one of this litter and all of the other survived and were sacrificed on the 42nd day after bilateral adrenalectomy. In all the rats that were sacrificed accessory adrenal bodies were found. Apparently, one litter was from a stock in which existence of accessory adrenal bodies was relatively rare while the other came of a stock with high incidence of accessories. In all the animals that died of adrenal insufficiency, within the usual period of survival, the characteristic globular predentin was found in the incisors. In the others, the incisors presented the rachitic-like changes. It is tempting to suppose that a disturbance in calcium metabolism induced by adrenal insufficiency is a factor in the development of rickets, in these animals, but without further proof this would be mere speculation.

It is of considerable interest that the characteristic response to adrenalectomy (globular predentin), in rats, was also observed incidentally on three rats receiving large doses of parathyroid extract. They were permitted to survive 19 hours. The globular predentin, found in the rat incisor after administration of the extract, is shown in figure 2. The photomicrograph (no. 3) shows a longitudinal section of an upper incisor at the distal end of the organic enamel matrix. The rat was given a single injection of 75 units of parathyroid extract and was sacrificed at the end of 19 hours. The late predentin, *L*, is sprinkled with globules, *Gl*, which stain like intermediate dentin. Comparison of this photomicrograph (3) with the two upper ones (1, 2), in figure 2, shows the striking similarity between the changes caused by the action of parathyroid extract and those due to adrenalectomy. Further studies on this question are necessary. However, this observation lends support to the suggestion made by one of us (Rogoff, 1934), based upon other observations, that a

probable functional interrelationship exists between the adrenal and parathyroid glands. Indeed, it is possible that the parathyroid may play a rôle in the production of globular predentin found in the incisor of the adrenalectomized rat.

## SUMMARY

Complete adrenalectomy, in the rat, causes disturbance in calcification of dentin. Characteristic changes occur in the predentin of the incisors. These changes are manifested by the presence of globules disseminated throughout the predentin of the middle third of the incisor.

The "globular predentin" stains, with eosin and hematoxylin, like intermediate dentin. It was not present in nearly a thousand rats that were studied in other investigations, in which adrenalectomy was not performed, except in three animals that were given single doses of parathyroid extract and examined 19 hours later.

Other evidences of disturbed calcification in the dentin are the deep staining of the labial dentin by hematoxylin and prominent stratification in the lingual dentin. In adrenalectomized animals that survived up to about ten days the post-operative dentin could be distinguished from the pre-operative by the presence of a deeply stained band corresponding to the portion of dentin that was laid down and calcified about the time of the operation. The presence of this band permits measurements of the post-operative dentin from which can be calculated the survival period of the animal.

In adrenalectomized rats whose survival was prolonged by the presence of accessory adrenal bodies, the characteristic globular predentin was absent. They showed changes similar to those found in rickets, viz., wide predentin (40–84 $\mu$) and prominent interglobular dentin. This observation suggests the probability of a relation of disturbed adrenal function to the calcium disturbances associated with the development of rickets.

The results obtained in these experiments afford interesting information on calcification processes of enamel and dentin. In a small number of animals the enamel-forming cells (ganoblasts) showed intracellular globules which stained deeply with hematoxylin.

Occurrence of globular predentin in the three animals that were subjected to the action of parathyroid extract confirms the observation that adrenal insufficiency is associated with disturbances in calcium metabolism (Rogoff and Stewart, 1928). It also lends support to the suggestion of a functional interrelationship between the adrenal and parathyroid glands. Although evidence favors the probability that the adrenal cortex is primarily involved, the possible relation of the medulla has not been excluded in these experiments.

## REFERENCES

BECKS, H. AND W. B. RYDER. Arch. Path. **12:** 358, 1931.
ERDHEIM, J. Mitteilungen aus den Grenzgebieten **16:** 632, 1906.
GOLDBLATT, H. Ergebn. allg. Path. u. path. Anat. **25:** 58, 1931.
ROGOFF, J. M. Science **80:** 319, 1934.
ROGOFF, J. M. AND G. N. STEWART. This Journal **86:** 25, 1928.
SCHOUR, I. AND M. C. SMITH. Proc. Soc. Exper. Biol. Med. **32:** 1, 1934.
SCHOUR, I., W. R. TWEEDY AND F. A. MCJUNKIN. Am. Journ. Path. **10:** 321, 1934.
SCHOUR, I. AND H. B. VAN DYKE. Am. Journ. Anat. **50:** 397, 1932

# ELECTROLYTE CHANGES IN MUSCLE DURING ACTIVITY

WALLACE O. FENN AND DORIS M. COBB

*From the Department of Physiology, School of Medicine and Dentistry, The University of Rochester, Rochester, N. Y.*

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While changes in the organic chemistry of muscles in activity have been intensively studied there are relatively few observations on changes in the inorganic composition and no agreement as to the facts. In perfused frog muscle Mitchell and Wilson (1921) found no loss of potassium except during stimulation to exhaustion. Ernst and his collaborators found a loss of potassium by direct stimulation but not by indirect stimulation (Ernst and Scheffer, 1928; Ernst and Csúcs, 1929) but later concluded that when the permeability was increased by high potassium content of the perfusate (Ernst and Fricker, 1934) there was a loss of potassium even with indirect stimulation. Mond and Netter (1930) also found no loss of potassium in frog muscle. In human muscles Ewig and Wiener (1928) present evidence that potassium and phosphate are increased in the blood after exercise while Dill, Talbot and Edwards (1930) find only an increase in phosphate in the blood and a loss of an ultrafiltrate of plasma which goes into the muscles. Baetjer (1935) has noted an increase in potassium in the blood of cats after exhausting stimulation. Kehar and Hooker (1935) have shown a loss of potassium from heart muscle during ventricular fibrillation.

In only one of these studies was the muscle itself analyzed for potassium. In some cases the muscles were artificially perfused and were abnormal due to the swelling as indicated by the high sodium and chloride contents (Ernst and Csúcs, 1929). Experiments were therefore undertaken to measure the potassium content of frog and rat muscles after stimulation with the muscle in the body with its normal blood supply intact. The results show a small loss of potassium from frog muscle in severe fatigue and in rat muscle a somewhat greater loss of potassium (accompanied by a gain of chloride, sodium and water) which is largely reversible during recovery.

METHODS. Samples weighing 200 to 500 mgm. were dried at 100°C. to constant weight for water content. Potassium and sodium were analyzed after dry-ashing overnight in platinum crucibles at 500°C. in an electric furnace. Samples weighing 200 to 300 mgm. were used. Sodium was

analyzed by Salit's (1932) method and potassium by a modification of the method of Shohl and Bennett (1928). In the latter procedure the potassium precipitation was rendered more complete by exposure to 100°C. (suggestion of Dr. A. H. Hegnauer) and the precipitate was separated by centrifuge (suggestion of Dr. J. I. Thaler). Larger samples of about 1 gram were necessary for chloride analyses by the method of Van Slyke (1923). Wet weights were obtained quickly on a torsion balance.

*Frog muscle.* Three series of experiments were performed with slightly varying techniques. *Series 1, control:* A frog was decerebrated by crushing the brain with a strong hemostat; one leg was tied off at the knee or thigh with a strong ligature; muscle samples were weighed out for analysis for water and potassium contents; the other leg was similarly tied off and sampled in the same way. *Series 2, indirect:* After decerebration sciatic nerves were exposed on both sides, either in the thigh or the abdomen (through the back). Both sciatics were cut and one of them was stimu-

TABLE 1

*Stimulation of frog muscle*

(Water and potassium contents per 100 grams dry weight)

| SERIES | NUMBER OF MUSCLES | NUMBER OF FROGS | RESTING MUSCLE | | AVERAGE CHANGE DURING STIMULATION | | |
|---|---|---|---|---|---|---|---|
| | | | K | $H_2O$ | K | $H_2O$ | P.e. |
| | | | *mM* | *cc.* | *mM* | *cc.* | *mM* |
| 1. Control | 14 | 3 | 46.6 | 395 | 0.05 | 3 | ±0.22 |
| 2. Indirect | 21 | 8 | 46.0 | 379 | −0.26 | +121 | ±0.27 |
| 3. Direct | 11 | 6 | 46.2 | 384 | −2.6 | +81 | ±0.25 |

lated *indirectly* at just threshold strength, either by rhythmical short tetani from an induction coil or by condenser charges at rates varying from 10 to 400 per minute. Stimulation was continued from 10 to 30 minutes and fatigue became pronounced in most cases. Kymograph records were usually taken of the contraction of the gastrocnemius muscle using an isometric lever. Immediately after stimulation the animal was bled to death and comparable samples from stimulated and unstimulated sides were taken for analyses. *Series 3, direct:* After decerebration, one leg was tied off at once and sampled while the other was stimulated *directly* by intermittent tetanus with electrodes on the knee and ankle. The muscle was kept completely fatigued for 10 minutes after which this leg also was tied off and comparable samples were taken for analysis.

The results of these three series of experiments are summarized in table 1. The average algebraic difference in the control series between right and left muscles was only 0.05 out of a total 46.6 millimols potassium, but the average difference (plus or minus) in potassium content between

any two matched muscles was 2 per cent. Series 2 showed slightly less potassium after stimulation but this change was statistically insignificant. In series 3 every stimulated muscle showed a loss of potassium averaging 2.6 millimols out of 46.2, this difference being ten times the probable error. This greater loss in series 3 is presumably due to the more excessive direct stimulation. Series 3 also differed from series 2 in that the control muscle was sampled before stimulation instead of afterwards, but this difference was probably not influential. It is evidently difficult, therefore, to show a loss of potassium in frog muscles after stimulation in situ unless the stimulation is applied directly to the muscle. The stimulated muscles always contained more water than the controls confirming in this respect the observations of Back, Cogan and Towers (1915).

*Rat muscles.* In a preliminary series of eight experiments (*series A*), rats were urethanized by 1.5 grams (in later experiments, 1.2 grams) per kilo body weight intraperitoneally. One sciatic was exposed and the severed end of the nerve was pulled inside a glass tube within which it

TABLE 2

*Stimulation in rat muscles. Series A*

(Millimols or cubic centimeters per 100 grams dry weight)

| SUBSTANCE | NUMBER OF MUSCLES | AVERAGE RESTING COMPOSITION | AVERAGE CHANGE IN STIMULATION |
|---|---|---|---|
| K | 19 | 45.7 mM | −3.34 mM |
| Cl | 16 | 5.28 mM | +2 37 mM |
| $H_2O$ | 9 | 310 cc. | +48 cc. |

made contact with the electrodes; it was stimulated for 5 to 30-minute periods by brief tetani, usually delivered once a second from a Harvard induction coil. Immediately after stimulation the animal was bled to death and symmetrical muscles were sampled for potassium, chloride and water. Unfortunately different muscles were usually used for these three substances so that the figures are not entirely comparable.

The results are briefly summarized in table 2. Potassium was lost in significant amounts and chloride and water were gained, all the concentrations being compared on the basis of dry weights. The gain in water content averaged 48 cc. per 100 grams of dried muscle (water content of the fresh muscle increased from 75.6 per cent to 78.2 per cent). The gain in chloride was 2.37 millimols per 100 grams. In the plasma this amount of chloride is associated with only 28 cc. of water so that if the chloride in the muscle is extracellular and has the same concentration as in plasma, 48 minus 28, or 20 cc. of water must have gone into the cells.

A similar conclusion is reached from the data of figure 1 obtained from a later series of experiments (*series B*) like the last except that the muscles

were stimulated for 30 minutes by unidirectional condenser charges at frequencies varying from 23 to 1480 per minute. The upper graph shows the increase in the water content of the muscles per 100 grams dry weight. In some of these muscles chloride contents were also determined. From the increased chloride content the extracellular water is calculated assuming 8.93 cc. water per millimol of chloride, this being the proportion found in the plasma. On the average (13 cases) only 42 per cent of the water gained by stimulation is extracellular. The water gained in these experiments increases rapidly to a maximum at a low frequency and then remains constant or possibly decreases slightly at higher frequencies.

The graph is drawn to suggest a slight decrease largely because the data of figure 2 show this effect and because the potassium losses in the same

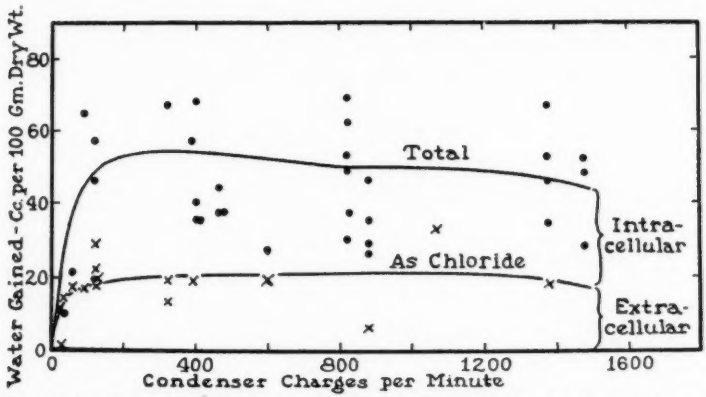


Fig. 1. Water and chloride gained by muscle when stimulated at different frequencies. The chloride is expressed as cubic centimeters of water in which it would be dissolved if in the same concentration as in plasma.

muscles were slightly less at higher frequencies than at medium frequencies. These potassium analyses in series B were made on 55 pairs of muscles from 22 rats. All but 4 pairs showed a loss of potassium on stimulation per 100 grams of dry weight. The individual variations were large; the average loss at each frequency is shown by crosses in figure 2. The average composition of the muscles of this series is shown in table 3, together with a few determinations on rat plasma. The average potassium content of the muscles was 47.3 millimols per 100 grams dry weight. The monthly averages (about 20 analyses each) beginning May 6 and ending November 6, were 45.7, 46.5, 48.3, 51.2 (August), 44.7, and 46.6, the probable error of each average being $\pm 0.5$. These figures may indicate, therefore, a seasonal increase in potassium content during the warm weather followed by a fall to 44.7 beginning September 19.

As a study of the effect of frequency of stimulation on electrolyte changes the experiments of series B were unsatisfactory because of a variety of minor changes in technique which were introduced from time to time. Consequently a final series of six experiments (*series C*) was performed with attention to uniformity, particular care being taken to keep the stimulus barely maximal to avoid injury to the nerve and consequent nerve fatigue. The threshold stimulus was about 0.025 microfarad and 0.8 volt.

It was found that the gastrocnemius and semimembranosus muscles gave the most uniform results and these only are reported. The tibialis and biceps femoris muscles which were also analyzed gave, in general,

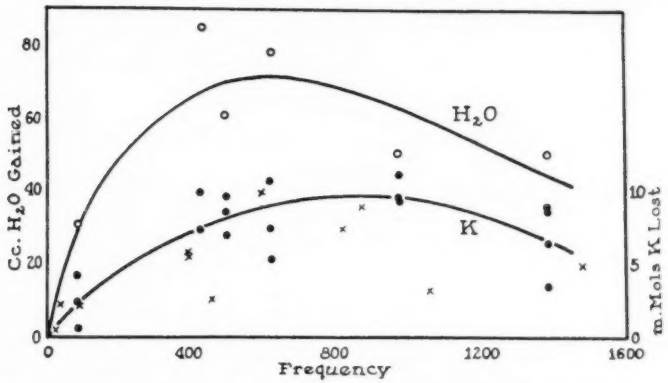


Fig. 2. *Lower curve.* Loss of potassium in milli-equivalents per 100 grams dry weight due to stimulation, drawn to follow the dots. Dots: individual values from gastrocnemius and semimembranosus muscles. Crosses: average losses from gastrocnemius, tibialis, biceps femoris and semimembranosus muscles in an earlier less uniform series of experiments. *Upper curve.* Gain in water in cubic centimeters per 100 grams dry weight. Average values from muscles plotted as dots in the lower curve.

similar results but occasionally showed very little change in stimulation. The latter muscle at least may have had its blood supply or nerve supply partly interfered with by the operation.

The results of experiments of series C are shown in figure 2. The upper graph shows the average water gain in all the determinations while the lower graph shows the individual potassium losses found in analysis of 18 pairs of muscles. Crosses on the lower graph are average results from muscles of series B, already described. From these results the slightly decreased electrolyte exchange at high frequencies seems to be confirmed.

The loss of potassium due to stimulation is largely compensated by a

gain in sodium. In 14 pairs of muscles, one stimulated for 30 minutes at 400 charges per minute and the other resting, analyses were made for both sodium and potassium. Calculating the results per 100 grams of dry weight as usual, the averages showed a gain of 8.3 millimols of sodium and a loss of 6.1 millimols of potassium. The average chloride gain from a similar stimulation was 2.8 millimols (11 pairs of muscles). If all this chloride were extracellular an equivalent amount of sodium must also have been extracellular, leaving 8.3 minus 2.8, or 5.5 millimols of sodium entering the cells to balance a loss of 6.1 millimols of potassium. So far as these results go, therefore, there is no need to assume (with Ernst and his collaborators, loc. cit.) that the loss of potassium on stimulation represents a new formation of potassium ions from bound potassium. The same is true in the data of Ernst and Csúcs (1929) (see their table 1). In the seven frog muscles analyzed by these authors there was a gain of 0.387

TABLE 3

*Average composition of rat muscle and plasma*

(Millimols)

| | NUMBER OF ANALYSES | MUSCLE | | PLASMA |
|---|---|---|---|---|
| | | Per 100 grams dry | Per 100 grams wet | Per 100 cc. |
| K | 113 | 47.3 ± 2.5 | 11.3 | 0.4 |
| Na | 20 | 7.6 ± 1.1 | 1.82 | 14.4 |
| Cl | 23 | 5.4 ± 0.7 | 1.29 | 10.8 |
| $H_2O$ | 33 | 318 ± 9 cc. | 76.1% | |

Probable error $= 0.6745 \sqrt{\frac{\Sigma d^2}{n}}$.

millimol of sodium and of 0.234 millimol chloride. The excess sodium is 0.153 millimol which partly balanced a loss of potassium of 0.272 millimol. The remainder of the potassium lost could be balanced by the loss of 0.127 millimol of phosphorus from the same muscles.

The time course of the electrolyte changes described for rat muscles has not been well worked out as yet. A series of experiments was tried with more or less uniform stimulation for 5, 10, 20, 30, and 60 minutes. All showed typical changes which were probably slightly lower on the average in the muscles stimulated for the shorter periods but the variations were too large to make this certain. After the first 5 minutes the change is probably slow.

In two experiments the rats were stimulated continuously for 3 hours before bleeding to death and sampling. In only one of these was the potassium loss appreciably larger than after 30 minutes. We were surprised to find, however, that the stimulated muscle of each pair was less

hydrated than the control, as shown by data for rats 1 and 2 in table 4. Evidently during this prolonged stimulation the animal became dehydrated and found it easier to get water from the stimulated muscle than from the resting muscles—probably because electrolytes could more easily escape with the water. Following this finding two more 3-hour experiments were tried in which water was supplied intraperitoneally at intervals (5 cc. in all) (rats 3 and 4, table 4). With this modification all the stimulated muscles were found more hydrated than their controls. This is also the invariable result after shorter periods of stimulation, as already mentioned. A few analyses for sodium showed that potassium largely escaped by exchange with sodium. The chloride changes were

TABLE 4

*Electrolyte changes in three-hour stimulation*

(M.mols and cc. $H_2O$ per 100 grams dry weight)

| | WITHOUT WATER | | | | | WATER SUPPLIED | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Rat 1 | | Rat 2 | | | Rat 3 | | | Rat 4 | | | |
| | K | $H_2O$ | K | Na | $H_2O$ | K | Cl | $H_2O$ | K | Na | Cl | $H_2O$ |
| Gastrocnemius | −2.1* | −5 | −17.0* | +12.9 | −6 | −17.4 | +1.6 | +28 | −13.9 | +12.1 | +0.6 | +24 |
| Tibialis | −4.3 | −23 | −19.4 | | −1 | | | | | | | |
| Biceps femoris | −6.3 | −3 | −24.7 | | −45 | −7.2 | +0.1 | +19 | −4.6 | +5.2 | +0.1 | +20 |
| Semimembranosus | −4.7* | −27 | −21.7 | +11.3 | −2 | | | | −13.6 | +11.4 | | +16 |

Frequency of stimulation 103, 400, 660 and 400 per minute, respectively, in rats 1 to 4.

All figures are differences between the resting and the stimulated muscle.

In no. 2, the biceps femoris lost 1.6 m.mols of chloride.

* Average of 2 analyses.

all comparatively small, there being as usual a small gain where water was supplied and in one analysis a slight loss (1.6 millimols) when water was withheld.

*Electrolyte changes in recovery.* A number of experiments were tried in which one leg was stimulated as usual and then the rat was allowed to recover for 1 to 2 hours before the muscles were sampled. The stimulated muscle still showed the usual electrolyte changes, a loss of potassium and a gain in sodium and chloride, though the differences seemed smaller. A better measure of recovery was found in experiments where both muscles were stimulated equally for 30 minutes. One leg was then tied off at once and the muscles sampled. The other leg was left in situ in the rat for a recovery period, usually lasting 2 hours, before it also was tied off

and sampled. This technique left the muscles more bloody than when the animal was bled to death. Most of the blood was removed, however, by blotting the samples rather vigorously on filter paper before weighing.

The chief difficulty in this technique lies in obtaining uniform stimulation of both legs. The method finally adopted was to expose both sciatics as usual and stimulate them alternately for 30 to 40 seconds with an interrupted current from the secondary of a Harvard induction coil, the stimulating electrodes being applied by hand. The right nerve was kept moist in the tissues while the left was being stimulated and vice versa. This stimulation was kept up for 30 minutes alternating each minute.

TABLE 5

*Electrolyte changes in gastrocnemius during recovery*

(M.mols or cc. per 100 grams dry weight)

| RAT NUMBER | RECOVERY PERIOD | K | Na | Cl | $H_2O$ |
|---|---|---|---|---|---|
| | *hours* | *mM* | *mM* | *mM* | *cc.* |
| 1 | 1.5 | +1.2 | −9.6 | −3.4 | −46 |
| 2 | 3.5 | +4.9* | | | −57 |
| 3 | 1.0 | +2.9* | | −0.6 | −33 |
| 4 | 2.0 | +3.3* | | | −46 |
| 5 | 0.5 | +2.1* | −4.4 | | −37 |
| 6 | 1.9 | +4.8 | −7.5 | | −46 |
| 7 | 1.5 | +5.9 | −4.3 | | −34 |
| Average | | +3.6 | −6.4 | −2.0 | −43 |
| Average during stimulation | | −6.1 | +8.3 | +2.8 | +49 |

Each figure represents the difference between a recovered and a control muscle, both having been stimulated. A plus sign indicates a gain during recovery and a minus sign a loss.

* Average of 2 samples.

The data on the gastrocnemius muscle are most complete and these are shown in table 5 for seven different rats.

The significance of the table is best seen from the average values given underneath, average values obtained in similar experiments during stimulation being included for the sake of comparison. The two sets of figures are not strictly comparable because they were obtained on different series of rats but a semiquantitative comparison seems justified. The figures indicate that all the changes observed during stimulation are one-half or three-quarters reversed in recovery of 1 to 3 hours' duration. The changes during recovery, like those during stimulation, indicate that the sodium in excess of chloride is just about sufficient to balance the potassium changes. The essential changes during stimulation are therefore: 1, an exchange of potassium and sodium, and 2, a gain of sodium chloride and water, both of which are reversed in recovery.

The average chloride loss in table 5 is based on only two divergent results but is supported indirectly by other analyses. The average chloride loss in four other analyses on muscles other than the gastrocnemius was 2.2 millimols.

In addition to the data of table 5, we have analyzed semimembranosus, tibialis and biceps femoris muscles from five rats. The average potassium changes in recovery in these three muscles were, a gain of 2.2 millimols in the semimembranosus, no change in the tibialis, and a negligible further loss of 0.9 millimol in the biceps femoris. Every gastrocnemius muscle and all but one of the semimembranosus muscles showed, therefore, a gain of potassium in recovery. Failure to recover in the other muscles may be due to the fact that the loss of potassium due to stimulation was less complete in these muscles immediately after the end of stimulation. These same muscles occasionally showed very small potassium losses during stimulation. It may also indicate less actual recovery, perhaps because of less adequate circulation in the more superficial muscles. A negative result of this sort does not detract, however, from the significance of the uniformly positive result found in the gastrocnemius where *potassium is repeatedly observed migrating from blood to muscle against a steep concentration gradient* in exchange for sodium.

Discussion. The changes which we have observed during stimulation are striking and consistent—a gain of sodium chloride and water and a loss of potassium in exchange for sodium—all of which are largely reversed in recovery. The intracellular water gain is due to an increase in osmotically active products inside the muscle (Hill and Kupalov, 1930; Meyerhof, 1930). The extracellular water gain may be due to increased filtration of plasma resulting from increased capillary pressure. The explanation of the exchange of potassium for sodium offers greater difficulty. If the muscle is normally permeable to potassium, then potassium could be lost by an acid-base change which would cause a greater increase in alkalinity in the muscle than in the plasma. A breakdown of phosphocreatine would accomplish this, but in the rather fatiguing stimulation employed in these experiments it seems more likely that the lactic acid formation is sufficient to cause an increase in acidity in the muscle. Furthermore such a mechanism offers no explanation for the entrance of sodium in nearly equivalent amounts. Moreover, if the normal muscle is regarded as impermeable to sodium (if not to potassium), then some increase in permeability seems required in the explanation. If now the permeability to sodium is increased during stimulation it is easy to understand that sodium enters and potassium leaves to some extent. (It is not necessary to suppose that the increase in permeability is enough to permit entrance of chloride also.) But this increase in permeability is only transitory, for only about 15 per cent of the potassium comes out.

Before recovery can even begin, therefore, the muscle must have regained its normal impermeability to sodium and this would make the observed loss of sodium in recovery impossible.

One way out of this dilemma is to suppose that only the surface of the fiber breaks down and exchanges its potassium for sodium, the lower layers being still impermeable to sodium, i.e., the effective membrane surface moves inwards. The fatigued fiber would then be in effect smaller in terms of dry weight than before. This is in agreement with the fact that the stimulated muscle, after 3 hours, is more dehydrated than the control. Recovery would then involve a process similar to that of hypertrophy where the muscle increases in size because the fibers increase in size rather than in number. In this way the sodium never really gets inside the membrane where it would be trapped and could not get out. Even this mechanism involves some difficulty for it has to be supposed that the degenerated superficial layers of the muscle which lose their potassium do not also lose their anions in exchange for chloride. Moreover, this growth of the fiber in recovery would require an intake of anions from these superficial layers through a membrane supposed to be anion impermeable. If the anion was phosphate this might occur if the phosphate entered in organic combination of some sort, rather than as a charged ion.

Further data are necessary before the complete electrolyte equilibrium in stimulation will be understood. Present indications are that the loss in total phosphorus is small.[1] The formation of lactate in the muscle and the dissociation changes of the buffer salts represent a more important item. It is still possible that sodium may enter one part of the muscle as sodium hydroxide to neutralize lactic acid while potassium may leave another part of the muscle with lactate or phosphate or in exchange for hydrogen. The results could be well explained by any mechanism which would draw potassium in one direction and sodium in the opposite direction. According to Keller (1935) this can be done by a difference of potential between the inside of the cell which is positive and the outside of the cell, which is negative, potassium going to the anode and sodium to the cathode. The source of this potential is, however, obscure and all the available evidence indicates that the inside of the cell is actually negative to the outside rather than positive.

Similar electrolyte changes occur in muscles as a result of injury. The fact that these stimulated rat muscles recover shows, however, that these are normal functional changes rather than irreversible injuries. Since they were stimulated indirectly there can be no direct injury to the muscles from the passage of the current. It should be emphasized also that the rat muscles, at least, showed these changes without being in any sense

[1] More recent analyses show that the same is true of Ca and Mg.

exhausted. The graphical records showed a prompt decrease in the height of contraction (tension) in the first minute but this soon reached a steady state at one-third or one-quarter the original tension where it remained with relatively little further decrease. The higher the frequency of stimulation the smaller the maximum tension reached at this fatigue level (cf. Davis and Davis, 1932). Much of the fatigue observed may be attributed to the nerve (cf. Heron, Hales and Ingle, 1934).

These results add further support to the theory that the muscle membrane is normally permeable to potassium. Otherwise the entrance of potassium into the fiber in recovery against the concentration gradient would be impossible to understand. Other evidence to the same effect has been presented from this laboratory both for frog muscles (Fenn and Cobb, 1934, 1935) and for cat muscles (Thaler, 1936).

The data presented suggest that electrolyte changes may be less at a stimulation frequency of 1480 than at 400. We feel, however, that more extensive data are needed to make this quite certain. If true it probably indicates that the nerve when stimulated at high frequency makes less effective demands upon the muscle than at more moderate frequencies. It is to be expected furthermore that the response of the muscle will reach a maximum at some frequency above which no further increase in intensity of response can be obtained. At a frequency of approximately 1000 per minute the responses of the gastrocnemius fuse to give a complete tetanus.

## SUMMARY

In rat muscles stimulation through the nerve causes an exchange of about 15 per cent of the potassium in the muscle for sodium, a gain of intracellular water, and a gain in extracellular water accompanied by sodium chloride. These changes are all largely reversible during 1 to 3 hours of recovery.

After 3 hours of continuous stimulation the fatigued muscle contains less water than the control unless water is supplied to the rat intraperitoneally during this period.

In frog muscles a loss of potassium was observed with certainty only when the muscle was stimulated directly.

## REFERENCES

BACK, M., K. M. COGAN AND A. E. TOWERS. Proc. Roy. Soc. s. B. **88**: 544, 1915.
BAETJER, A. M. This Journal **112**: 139, 1935.
DAVIS, H. AND P. A. DAVIS. This Journal **101**: 339, 1932.
DILL, D. B., J. H. TALBOT AND H. T. EDWARDS. J. Physiol. **69**: 267, 1930.
ERNST, E. AND L. CSÚCS. Pflüger's Arch. **223**: 663, 1929.
ERNST, E. AND J. FRICKER. Pflüger's Arch. **234**: 399, 1934.
ERNST, E. AND L. SCHEFFER. Pflüger's Arch. **220**: 655, 1928.
EWIG, W. AND R. WIENER. Ztschr. f. d. ges. exp. Med. **61**: 562, 1928.

FENN, W. O. AND D. M. COBB. J. Gen. Physiol. **17**: 629, 1934. This Journal **112**: 41, 1935.
HILL, A. V. AND P. S. KUPALOV. Proc. Roy. Soc. s. B. **106**: 445, 1930.
HERON, W. T., W. M. HALES AND D. J. INGLE. This Journal **110**: 357, 1934.
KEHAR, N. D. AND D. R. HOOKER. This Journal **112**: 301, 1935.
KELLER, R. Klin. Wchnschr. **14**: 945, 1935.
MEYERHOF, O. Biochem. Ztschr. **226**: 1, 1930.
MITCHELL, P. H. AND J. W. WILSON. J. Gen. Physiol. **4**: 45, 1921.
MOND, R. AND H. NETTER. Pflüger's Arch. **224**: 702, 1930.
SALIT, P. W. J. Biol. Chem. **96**: 659, 1932.
SHOHL, A. T. AND H. B. BENNETT. J. Biol. Chem. **78**: 643, 1928.
THALER, J. I. Proc. Soc. Exper. Biol. Med. **33**: 368, 1936.
VAN SLYKE, D. D. J. Biol. Chem. **58**: 523, 1923.

# STELLATE GANGLIA AND BREATHING[1]

JOHN M. BROOKHART, ELLIS H. STEFFENSEN AND ROBERT GESELL

*From the Department of Physiology, University of Michigan, Ann Arbor*

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Of the two innervations of the lungs, the control of breathing through the parasympathetics has received by far the greater attention. The striking effects of vagal section and of central stimulation and the ease of conducting these procedures readily account for the disproportionate accumulation of data. Only recently is more attention focusing on possible control of pulmonary ventilation through sympathetic innervation.

It is difficult to trace the fiber tracts through the stellate ganglia, and we have found no paper in comparative neurology dealing with the subject in the dog, but the anatomy is certainly compatible with functional nervous connection between the lungs and central respiratory mechanism via sympathetic fibers and severance of sympathetic connections with the lungs by stellectomy is accepted as a positive procedure.

Working on rabbits and cats, Barry (1913) found that blocking the trachea at the end of inspiration caused immediate relaxation of the respiratory muscles but only with the vagi intact. Mechanical asphyxia at the end of expiration produced a series of inspiratory efforts unaffected by vagotomy but somewhat reduced by combined vagotomy and stellectomy. In 1915 Boothby and Berry described apnea on pulmonary distention in vagotomized dogs. In 1921 Craigie found that reflex expiration and inhibition of inspiration produced by irritating gases were unaffected by vagotomy in the dog, and concluded that the provocative impulses probably travel in the sympathetics. About the same time Camis (1922) noted that reflex inhibition from central vagal stimulation of breathing in the cat and rabbit was augmented by stellectomy, suggesting reciprocal antagonism between vagal and sympathetic fibers. In 1924 Larsell and Burget working on the rabbit were unable to elicit bechic reflexes from mechanical and chemical irritation of the deeper air passages after double vagotomy. Possibly the anatomical differences between the dog and rabbit may account for discrepancies in the results of Craigie and of Larsell and Burget for pulmonary innervation is more complete in the dog. Employing pharmacological methods, McDowal (1926) found effects of vagal block diminished during sympathetic stimulation and increased

[1] This study was supported in part by a grant from the Rockefeller Foundation.

during sympathetic paralysis, indicating that the sympathetic system assumes control of breathing with vagi non-functional. Macklin (1929) proposed a hypothetic coördination of contraction of bronchial and respiratory muscles with important integrating pathways in either the vagus or sympathetic connections. In 1933 Cromer and Ivy found that removal of the stellate ganglia in the dog sensitized the central respiratory mechanism to vagus inhibition, sufficient at times to cause death with prolonged stimulation, a very rare occurrence with intact sympathetic innervation. Studies on chemical irritation of the pulmonary passages in the dog convinced Cromer, Ivy and Young (1933) that impulses inducing reflex effects travel in the vagi and in the sympathetics via the stellate ganglia. To quote "We do not believe that we have sufficient evidence to state that the impulses mediated by the stellate ganglia are specific in character, that is, that they are inspiratory-inhibiting or stimulating, or expiratory-inhibiting or stimulating. But we believe that it is clear from our observations that the respiratory center may be inhibited by impulses mediated by the stellate ganglia, and that in experiments dealing with the nervous control of respiration as influenced by stimuli acting in the lungs, the stellate ganglia as a pathway cannot be overlooked."

The importance of a possible antagonism between the sympathetics and parasympathetics under conditions more physiological than heretofore studied most certainly requires additional experiments. We have accordingly attempted a quantitative study with accurately graded physiological stimulation. We could think of no simpler and more precise method of such gradation than the application of intrapulmonary pressures below and above prevailing atmospheric pressures, producing lung volume changes sufficient to accelerate and inhibit respiratory rhythm. Curves of rate against pressure are readily established with the mercury buoyed spirometer and procedure described by Gesell and Moyer (1935). Such curves obtained under varying innervations served as indication of the extent of rhythm function exercised by the sympathetic and parasympathetic innervations of the lungs.

Weighting the spirometer provided intrapulmonary pressures greater than barometric and counterweighting provided pressures less than atmospheric. A graded set of weights, calibrated in terms of water pressure, giving 12, 24, 36 and 48 mm. negative pressures and 14, 28, 42 and 56 mm. positive pressures, were used in all experiments. Negative pressures were applied during expiration and positive pressures were applied at the onset of inspiration. A period of recovery was allowed after each reflex change in breathing during which normal rate of breathing was determined.

Average rate of normal breathing was determined over a period of thirty seconds to correct for sporadic changes in duration of individual

respiratory cycles. During altered breathing the rate was determined for the first respiratory cycle following application of positive or negative pressure. This procedure was necessitated by the accelerating effects of progressive asphyxia resulting from inhibited breathing. Since average rates were thereby prohibited, an uncontrollable divergence of curves

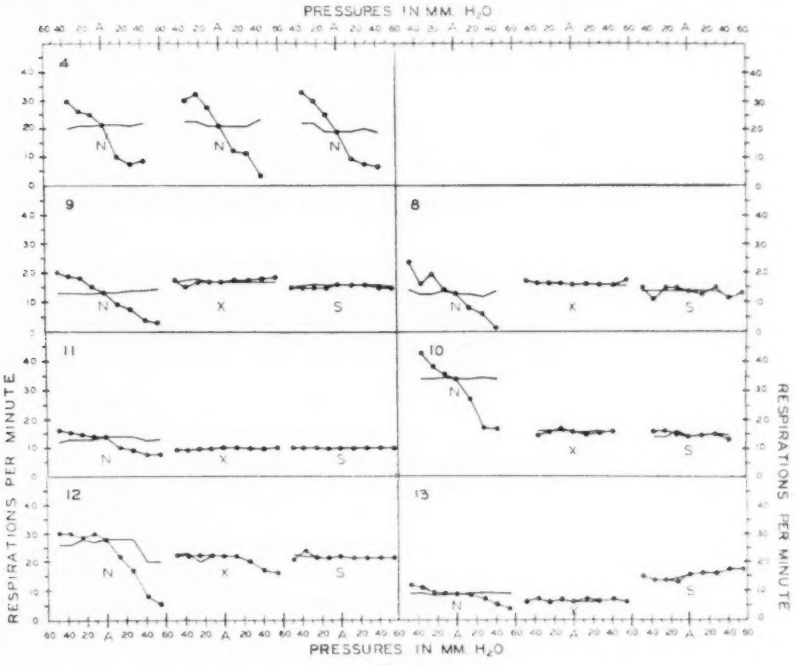


Fig. 1

incident to sporadic changes in rate of breathing was likely to occur, especially in lightly anesthetized animals. For that reason reproducibility of rate-pressure response against time was determined in several experiments.

There are 17 experiments in all (see figs. 1 and 2). In 5 of these, no. 4, 14, 15, 16, and 17, the relation of respiratory rhythm to pressure was determined for 3 complete series of pressure changes, with innervations intact. In the remaining experiments this relation was determined for only one series of pressure changes, with innervation intact. Ignoring sudden deflections of the curves, which most probably were due to reflex modification of breathing from various reflex stimulations (peristaltic wave, overfilled bladder, experimental procedures, etc.) the relation of

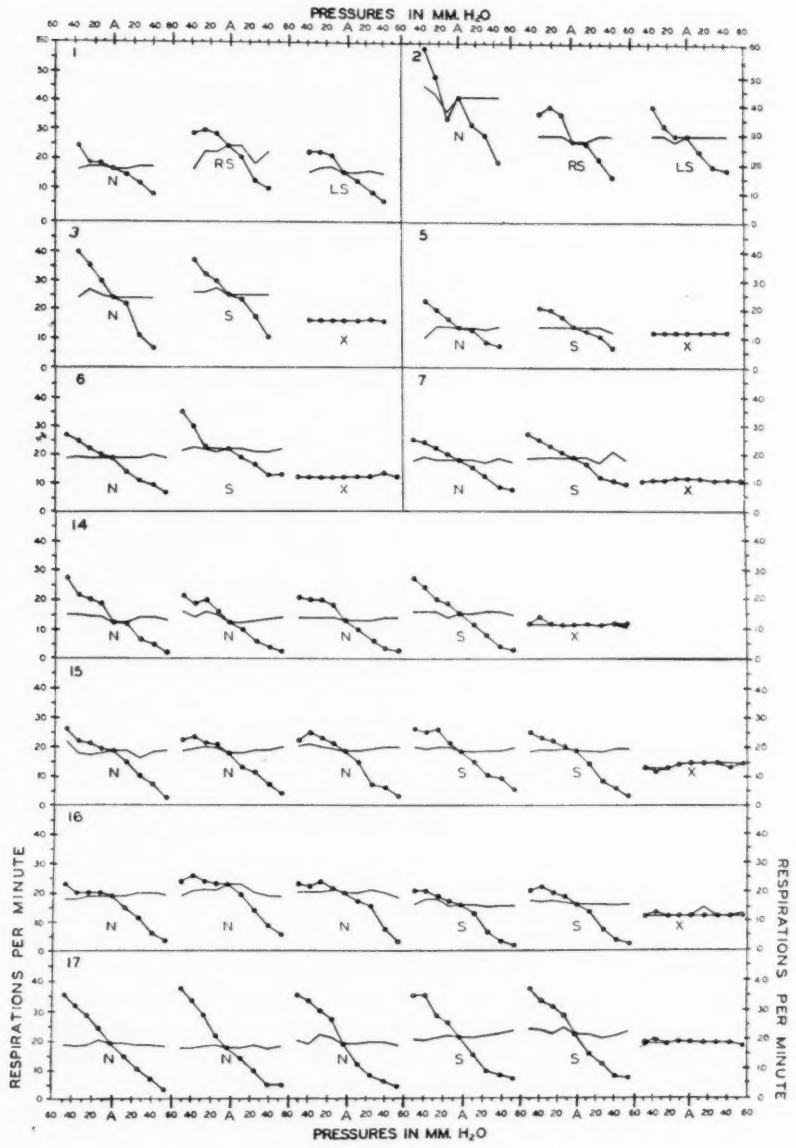
PRESSURES IN MM. H2O
RESPIRATIONS PER MINUTE
RESPIRATIONS PER MINUTE
PRESSURES IN MM. H2O
1
2
3
5
6
7
14
15
16
17
N
RS
LS
S
X

Fig. 2

rate to intrapulmonary pressure was roughly linear. In some animals the response to graded intrapulmonary pressure was greater than in others as indicated by the gradient of the curves. These differences may have been due to depth of anesthesia varying slightly from dog to dog or to other variation in excitability of the respiratory mechanism or to variations of distensibility of the lungs and torso.

A more careful inspection of results reveals a tendency toward flattening of curves in the greater negative pressure regions. See experiments 12, 14, 15 and 16. The reason for this is not entirely clear but interpreting the response to pressures on a lung-volume basis (Hammouda and Wilson, 1932; Gesell and Moyer, 1935) and bearing in mind the curvature of the ribs, it is not altogether unreasonable to assume that negative pressures produce smaller changes in lung volume than do positive pressures, hereby affecting the curve.

Whatever the cause of occasional flattening of the curves in the negative pressure region may be, the fact remains that reproducibility of response is sufficiently good, in fact better than anticipated for a mechanism so highly susceptible to various influences as the respiratory mechanism. It meets, very satisfactorily, the requirement of our present experiments.

We have studied the response of breathing to pressure with two sequences of denervation—vagotomy followed by stellectomy and stellectomy followed by vagotomy. The stellate ganglia were removed through the first intercostal space, which was approached through an incision along the posterior margins of the M. triceps brachii and M. deltoideus. The first sequence of which there are 6 experiments has the advantage of immediate demonstration of the adequacy of the method for detecting functional response. Vagotomy strikingly abolishes all signs of rate response to changes in pulmonary volume, thus supporting the general contentions, first, that lung volume effects are transmitted through the vagus nerves,

Figs. 1 and 2. Effects of vagotomy and stellectomy on the response of the respiratory mechanism to changes in intrapulmonary pressure (lung volume).

Rate per minute is plotted on the ordinates against pressure in millimeters of water on the abscissae. Pressures to the left of *A.* (atmospheric pressure) are negative, producing collapse of the lungs and pressures to the right of *A.* are positive, producing inflation.

The curves marked with solid circles show the response to pressure changes. The uninterrupted curves indicate the corresponding rates at atmospheric pressure. *N* indicates results with intact innervation, *X* after double vagotomy, and *S* after double stellectomy. *L.S.* indicates left stellectomy. *R.S.* indicates right stellectomy. Experiment 4 of figure 1 shows reproducibility of results with intact innervation. Experiments 8, 9, 10, 11, 12 and 13 show results with vagotomy preceding stellectomy. Experiments 1 and 2 of figure 2 show results with stellectomy, and experiments 3, 5, 6, 7, 14, 15, 16 and 17 with stellectomy preceding vagotomy. Experiments 14, 15, 16 and 17 also show reproducibility of results against time with innervation intact.

and second, that they are not transmitted through the sympathetics. These views are further strengthened by the finding that double stellectomy following vagotomy in no way altered the response to pressure changes following vagotomy alone.

In the light of recent experiments by Gesell and Moyer (1935) it is now apparent that the second contention is somewhat precarious. One need only compare the marked acceleration during central stimulation of the saphenous nerve, vagal innervation intact, with the decidedly lesser effects during vagal block to see the danger of such deduction. From such findings one may infer that absence of effects of double stellectomy after vagotomy is necessarily no sure index of possible augmenting action of the stellates when co-functioning with the important ancillary vagal component.

Conclusions are more significant in the second sequence of denervation where the ganglia are removed with vagal innervation still intact. Here, too, we are unable to see any effect of stellectomy on the response to graded pressure. Subsequent vagotomy, as in the other sequence of denervation, abolished rate response to lung-volume changes. We are, therefore, forced to conclude that under the conditions here described lung-volume reflex effects on rate of breathing are transmitted primarily through the vagus nerves and that impulses passing through the stellate ganglia contribute no detectable changes in rate. While our experiments seem to eliminate the stellate ganglia as mediators of proprioceptive impulses so easily demonstrated for the vagus nerves, they in no way contradict the findings of Camis and of Ivy and Cromer. It is not improbable that extended studies along other lines of approach may reveal some functions, still unknown, of the sympathetic innervation of the lungs.

## SUMMARY AND CONCLUSIONS

The influence of the sympathetic innervation of the lungs and the interaction of the sympathetics and parasympathetics in the control of breathing was reviewed.

Important effects of stellectomy under artificial conditions indicated the need of study of sympathetic function under conditions more physiological than heretofore attempted.

The response of respiratory rhythm to graded physiological stimulation (changes in intrapulmonary pressures above and below atmospheric pressure) served as the measure of rhythmic function under varying innervations.

With innervation intact, rate was roughly linear to pressure (lung-volume).

Reproducibility of results against time during intact innervation warranted comparison of rate-pressure curves under modified innervations.

Two sequences of denervations were studied—vagotomy followed by stellectomy and stellectomy followed by vagotomy.

Vagotomy abolished lung-volume-rhythm reflexes. Subsequent stellectomy was without further effects.

Stellectomy with vagal innervation intact had no demonstrable effect on lung-volume-rate reflexes. Subsequent vagotomy abolished these reflexes.

Abolition of lung-volume-rate reflexes by vagotomy supports accepted opinion that such proprioceptive reflexes are mediated primarily through the vagus nerves. It is insufficient proof that rate effects are not also mediated through the stellate ganglia, for the vagus nerves are ancillary to other rate provoking mechanisms.

The absence of change in lung-volume-rate response following stellectomy with ancillary vagal function intact, however, indicates that the stellate ganglia are in all probability not concerned with the mediation of proprioceptive respiratory reflexes.

## REFERENCES


BARRY, D. T. J. Physiol. **45**: 473, 1913.
BOOTHBY, W. M. AND F. B. BERRY. This Journal **37**: 433, 1915.
CAMIS, M. Arch. di Fisiol. **21**: 385, 1922.
CRAIGIE, E. H. This Journal **59**: 346, 1922.
CROMER, S. P. AND A. C. IVY. This Journal **104**: 457, 1933.
CROMER, S. P., R. H. YOUNG AND A. C. IVY. This Journal **104**: 468, 1933.
GESELL, R. AND C. MOYER. Quart. J. Exper. Physiol. **25**: 13, 1935.
HAMMOUDA, M. AND W. H. WILSON. J. Physiol. **74**: 81, 1932.
LARSELL, O. AND G. E. BURGET. This Journal **70**: 311, 1924.
MCDOWAL, R. J. S. Quart. J. Exper. Physiol. **16**: 291, 1926.
MACKLIN, C. C. Physiol. Rev. **9**: 1, 1929.

# CREATINURIA INDUCED BY THE INGESTION OF GLUCOSE AND FRUCTOSE AND BY EXERCISE

JOHN HALDI AND GEORGE BACHMANN

WITH THE ASSISTANCE OF W. WYNN AND J. M. LITTLE

*From the T. T. Fishburne Laboratory of Physiology, Emory University, Emory University, Ga.*

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Some years ago Cathcart (1909) observed that the elimination of creatine in the urine induced by fasting fell at once when the fast was broken with a carbohydrate diet, but increased with a fat diet. Mendel and Rose (1911) from similar observations concluded that carbohydrates are indispensable for normal creatine-creatinine metabolism. They pointed out that creatine appears in the urine under all conditions which interfere with the glycogenic function of the liver or which render the organism unable to oxidize sugar. Rose (1916) later reported additional evidence showing that creatine elimination results from a deficiency in carbohydrate utilization.

In a series of experiments in which we have studied the metabolism of sugars both with the subject at rest and taking exercise, we have found that creatine excretion may be induced by 1, the ingestion of glucose and fructose taken either separately or together; 2, exercise preceded by ingestion of water; 3, exercise preceded by ingestion of the aforesaid sugars. Under each of these conditions we have invariably found (Bachmann and Haldi, 1935) in keeping with the observations of other workers (Deuel, 1929; Carpenter and Fox, 1931) a marked increase in carbohydrate metabolism.

METHOD. The subjects of these experiments were male adults in good physical condition. There was a marked difference in their physical build, one being 182 cm. in height and weighing approximately 80 kilos, and the other 170 cm., weighing 55 kilos. On the day of the experiment the subject rode to the laboratory between 7:30 and 8:00 a.m., without breakfast, the last meal having been taken no later than 7 o'clock the evening before. After reclining on a couch for at least 45 minutes the subject arose, voided urine, and then immediately resumed the recumbent position for another 45 minutes, after which he again voided urine. This second 45 minute period shall be referred to hereafter as the post-absorptive period. Fifty grams glucose, 50 grams fructose, or a mixture of 25 grams glucose and 25 grams fructose dissolved in 500 cc. of water at 37°C. were then in-

gested. A number of control experiments were run in which 500 cc. of water were ingested at the same temperature as the sugar solutions. After the ingestion of water or one of the above mentioned sugar solutions the subject either reclined on the couch for one and three quarters hours in the rest experiments, or pedalled a bicycle ergometer for 30 minutes in the exercise experiments against a tension of 2.5 kilos. The rate of pedalling was so adjusted by timing with a metronome, that the work per minute amounted to approximately 550 kilogram-meters. Immediately after the exercise the subject alighted from the bicycle onto an adjacent couch and reclined for one and one-quarter hours.

At the end of the experiment the bladder was emptied for the final collection of urine. The urine voided after the preliminary rest period was discarded, while the second and third collections were analyzed for creatinine and creatine. Analyses of preformed creatinine were made by the Folin method (1914) with 10 cc. of urine in a 500 cc. flask (Hawk and Bergeim, 1931). Creatine as creatinine was determined by the same colorimetric method after boiling the acidified urine with granulated lead on a hot plate (Benedict, 1914). The picric acid used in the analyses was purified according to Benedict's directions (1929). The urine voided at the end of the post-absorptive period will be referred to in this paper as the "post-absorptive" sample and that which was collected at the completion of the experiment as the "post-experimental" sample. The analytical data of the post-absorptive and post-experimental samples are tabulated in the P-A and P-E columns respectively of tables 1 and 2. A variable length of time (50 to 74 minutes) elapsed in the different experiments between the first urination and the collection of the post-absorptive urine. In most of the experiments the time interval was 55 minutes to 1 hour. The time elapsing between the post-absorptive and post-experimental collection varied from 1 hour and 53 minutes to 2 hours. The hourly excretion of creatine, creatinine and preformed creatinine plus creatine as creatinine is given in tables 1 and 2.

*Creatine excretion. Rest experiments.* Creatine was excreted during the post-absorptive periods in only 3 out of 33 experiments. In the 8 control experiments of table 1 with the subjects at rest throughout the experiment, there was no creatine excretion either during the post-absorptive period or during the rest period of nearly two hours following the ingestion of 500 cc. of water. In the remaining 25 rest experiments in which the sugars were ingested, creatine was found in the post-experimental urine in 16 experiments or 64 per cent of the cases. In experiments 23 and 62 there was a small amount of creatine excreted during the post-absorptive period, but in each of these experiments the rate of creatine excretion after ingestion of the sugars was more than two and one-half times greater than during the post-absorptive period. The average hourly excretion, as shown

in table 3, was higher following the ingestion both of fructose and a mixture of the sugars in equal parts than after the ingestion of glucose.

*Exercise experiments.* During the post-absorptive periods preceding exercise, creatine was excreted in only one out of 27 experiments with sugar and in none of the 8 control experiments in which water alone was taken

TABLE 1

*Experiments with the subject at rest*

| SUBSTANCE INGESTED | NO. OF EXP'TS. | NO. OF EXP'TS. WITH CREATINE IN URINE | | HOURLY EXCRETION MGM. CREATINE (AS CREATININE) | | HOURLY EXCRETION MGM. CREATININE (AVERAGE) | | HOURLY EXCRETION MGM. PREFORMED CREATININE PLUS CREATINE AS CREATININE (AVERAGE) | |
|---|---|---|---|---|---|---|---|---|---|
| | | P-A* | P-E† | P-A | P-E | P-A | P-E | P-A | P-E |
| Subject W. W. | | | | | | | | | |
| 500 cc. $H_2O$ (control exp'ts.) | 4 | 0 | 0 | 0 | 0 | 62.08 | 59.04 | 62.08 | 59.04 |
| 50 grams glucose | 5 | 0 | 4 | 0 | 1.38 one exp't.<br>0.81 one exp't.<br>3.38 one exp't.<br>1.30 one exp't. | 59.39 | 63.00 | 59.39 | 64.58 |
| 50 grams fructose | 6 | 1 | 3 | 1.57 (exp't. 23) | 4.26 one exp't.<br>3.10 one exp't.<br>4.15 exp't. 23 | 59.34 | 60.16 | 59.60 | 62.08 |
| 25 grams glucose and 25 grams fructose | 3 | 0 | 2 | 0 | 3.79 one exp't.<br>2.03 one exp't. | 63.27 | 67.79 | 63.27 | 69.73 |
| Subject J. H. | | | | | | | | | |
| 500 cc. $H_2O$ (control expt's.) | 4 | 0 | 0 | 0 | 0 | 82.05 | 80.64 | 82.05 | 80.64 |
| 50 grams glucose | 5 | 0 | 3 | 0 | 1.84 one exp't.<br>5.56 one exp't.<br>1.83 one exp't. | 84.11 | 77.25 | 84.11 | 79.09 |
| 50 grams fructose | 4 | 0 | 2 | 0 | 7.83 one exp't.<br>2.64 one exp't. | 83.05 | 78.31 | 83.05 | 80.93 |
| 25 grams glucose and 25 grams fructose | 2 | 1 | 2 | 0.73 (exp't. 62) | 3.99 one exp't.<br>2.29 exp't. 62 | 75.29 | 76.53 | 75.66 | 79.67 |

* P-A = post-absorptive sample. Urine excreted over a period of 50 to 74 minutes.

† P-E = post-experimental sample. Urine excreted over a period of 1 hour 53 minutes to 2 hours.

before the exercise. In the control experiments creatine was excreted in appreciable amounts during exercise and recovery in 3 out of 4 experiments with subject W. W. and in 2 out of 4 experiments with subject J. H., a total of 5 out of 8 experiments or 63 per cent. When the sugars were ingested creatine was excreted in 17 out of 19 experiments during exercise

and recovery, or 89 per cent of the cases. This is 26 per cent higher than in the exercise experiments when water alone was taken and 25 per cent higher than in the rest experiments when the sugars were ingested. In experiment 46 creatine was present in the post-absorptive urine but

TABLE 2

*Experiments with the subject taking exercise on a bicycle ergometer (550 kgm-m. of work per min. for 30 min.)*

| SUBSTANCE INGESTED | NO. OF EXP'TS. | NO. OF EXP'TS. WITH CREATINE IN URINE | | HOURLY EXCRETION MGM. CREATINE (AS CREATININE) | | HOURLY EXCRETION MGM. CREATININE (AVERAGE) | | HOURLY EXCRETION MGM. PREFORMED CREATININE PLUS CREATINE AS CREATININE (AVERAGE) | |
|---|---|---|---|---|---|---|---|---|---|
| | | P-A* | P-E† | P-A | P-E | P-A | P-E | P-A | P-E |
| **Subject W. W.** | | | | | | | | | |
| 500 cc. $H_2O$ (control expt's.) | 4 | 0 | 3 | 0 | 1.62 one exp't.<br>3.16 one exp't.<br>1.33 one exp't. | 52.88 | 55.57 | 52.88 | 57.10 |
| 50 grams glucose | 4 | 1 | 3 | 1.59 (exp't. 46) | 1.23 one exp't.<br>1.86 one exp't.<br>2.25 exp't. 46 | 52.95 | 53.15 | 53.35 | 54.51 |
| 50 grams fructose | 3 | 0 | 3 | 0 | 2.46 one exp't.<br>2.33 one exp't.<br>3.32 one exp't. | 54.27 | 56.11 | 54.27 | 58.81 |
| 25 grams glucose and 25 grams fructose | 3 | 0 | 3 | 0 | 0.73 one exp't.<br>4.45 one exp't.<br>1.77 one exp't. | 66.98 | 64.57 | 66.98 | 66.89 |
| **Subject J. H.** | | | | | | | | | |
| 500 cc. $H_2O$ (control expt's.) | 4 | 0 | 2 | 0 | 4.29 one exp't.<br>1.45 one exp't. | 69.81 | 71.42 | 69.81 | 74.29 |
| 50 grams glucose | 3 | 0 | 3 | 0 | 0.80 one exp't.<br>2.31 one exp't.<br>4.42 one exp't. | 74.37 | 78.14 | 74.37 | 80.65 |
| 50 grams fructose | 3 | 0 | 3 | 0 | 2.53 one exp't.<br>5.99 one exp't.<br>7.32 one exp't. | 71.91 | 69.77 | 71.91 | 75.04 |
| 25 grams glucose and 25 grams fructose | 3 | 0 | 2 | 0 | 6.44 one exp't.<br>4.72 one exp't. | 77.53 | 84.59 | 77.53 | 90.17 |

* P-A = post-absorptive sample. Urine excreted over a period of 50 to 74 minutes.

† P-E = post-experimental sample. Urine excreted over a period of 1 hour 53 minutes to 2 hours

the rate of excretion during exercise and recovery was one and one-half times greater than during the post-absorptive period. It should be noted that in the exercise, as in the rest experiments, the average excretion was greater when fructose or a mixture of the sugars was ingested than when

glucose alone was taken. We are unable to account for this difference in the effects of glucose and fructose.

The more frequent appearance of creatine in the urine with a combination of sugar ingestion and exercise than with either sugar ingestion or exercise alone would suggest a cumulative effect of the sugars and exercise. The same impression is obtained if the hourly excretions in all the rest experiments with the various sugars are added and averaged and this average compared with the average hourly excretion derived in the same way from the exercise experiments with the sugars. In the exercise experiments with sugar, the rest experiments with sugar, and the exercise experiments with water, the average hourly excretion of W. W. was 2.04, 1.72 and 1.53 mgm., and that of J. H. 3.83, 2.36 and 1.44 mgm., respectively.

TABLE 3

*Average increase in hourly excretion of creatine during the post-ingestion periods*

| INGESTED SUBSTANCE | SUBJECT | REST EXPTS. | EXERCISE EXPTS. |
|---|---|---|---|
| | | *mgm. per hour* | *mgm. per hour* |
| 500 cc. $H_2O$ | W. W. | 0 | 1.52 |
| | J. H. | 0 | 1.44 |
| 50 grams glucose in 500 cc. $H_2O$ | W. W. | 1.38 | 0.94† |
| | J. H. | 1.85 | 2.51 |
| 50 grams fructose in 500 cc. $H_2O$ | W. W. | 1.66* | 2.70 |
| | J. H. | 2.62 | 5.27 |
| 25 grams glucose and 25 grams fructose in 500 cc. $H_2O$ | W. W. | 1.94 | 2.32 |
| | J. H. | 3.14 | 3.72 |

* The absolute average excretion was 1.92 mgm. per hour.

† The absolute average excretion was 1.34 mgm. per hour.

These averages, however, are misleading for the cumulative effect of the sugars and exercise does not appear if the rest and exercise experiments with the sugars are averaged for each kind of sugar separately, as shown in table 3. The average excretion with subjects W. W. and J. H. in the exercise experiments when water alone was ingested, was respectively 1.52 and 1.44 mgm. creatine per hour, and 1.38 and 1.85 mgm. per hour when glucose was taken with the subject remaining at rest. When the ingestion of glucose was combined with exercise the average excretion was actually less with W. W. and only 0.70 mgm. greater with J. H. than when glucose was taken without exercise. When exercise was combined with the ingestion of a mixture of the sugars, the creatine excretion was far short of the sum of the effects produced separately by exercise and the ingestion of the sugars. In the case of fructose, a combination of exercise and ingestion

of the sugar resulted in creatine excretion in one subject in excess of the sum of the effects produced by exercise and ingestion of the sugar separately.

*Creatinine excretion.* In view of the prevailing opinion that in the organism creatinine is derived from creatine, we have included in the tables the hourly creatinine excretion to determine whether or not the excretion of creatine was associated with a corresponding diminution in the excretion of creatinine. It is possible that the ingestion of sugar or exercise might affect creatine-creatinine metabolism in such a way as to prevent the conversion of creatine into creatinine, in which event one would expect to find a diminution in creatinine excretion when creatine appeared in the urine. Instead of a decrease, however, there was a slight increase in most of our experiments, as shown in tables 1 and 2. In those experiments with sugar in which the excretion was lower in the post-experimental than in the post-absorptive urine, the differences were probably insignificant since they were of the same order as those of the control experiments with water. Nevertheless, we have made a statistical analysis of these results, recognizing, however, the restrictions of this method when applied to the limited data at our disposal. The probable error of the difference between the creatinine excretion during the post-absorptive and post-ingestion periods has been calculated and in no instance did the difference approach a value equal to three times the probable error of the difference. In all cases except one, the difference was less than twice its probable error. It may therefore be deduced that the urinary excretion of creatine in these experiments can not be attributed to an interference with creatine-creatinine metabolism.

Conclusion. Excretion of creatine in the urine has been noted in various conditions in which there is a deficiency in carbohydrate metabolism, such as fasting, phlorizin intoxication and diabetes. When induced by prolonged fasting, creatine was made to disappear by feeding either carbohydrate or protein (Rose, 1916). It has been shown that the cessation of creatine elimination with both kinds of feeding was due to the oxidation of carbohydrate; with protein feeding the carbohydrate was supplied by the sugar derived from the protein (Rose, 1916). These observations led Rose to the conclusion, based on the assumption that creatinine in the organism comes from creatine, that carbohydrate oxidation is essential for creatine-creatinine metabolism. He believes that with an impairment of carbohydrate metabolism some creatine escapes conversion into creatinine and is excreted in the urine.

In our experiments creatine excretion was induced by the ingestion of glucose and fructose taken separately or together and also by exercise, all of which conditions are conducive to a high carbohydrate metabolism. These observations do not necessarily invalidate Rose's theory but they

are difficult to reconcile with it. To ascribe both the presence and absence of creatine in the urine to the same factor, namely, carbohydrate metabolism, involves a contradiction. The contradiction, however, may be more apparent than real, for it is possible that in our experiments it was not the high carbohydrate metabolism itself, but some other process associated with it, that was responsible for the creatine excretion. We are not prepared at present to commit ourselves on the mechanisms that might account for the creatine excretion observed in these experiments.

## SUMMARY

The ingestion of 50 grams glucose, 50 grams fructose or a mixture of 25 grams of each of these two sugars led to the excretion of creatine in the urine of two normal male adults at rest in 16 out of 25 experiments.

There was a marked increase in carbohydrate metabolism following the ingestion of the sugars.

A higher average excretion of creatine was induced by fructose than by glucose or a combination of the sugars.

Exercise on a bicycle ergometer at the rate of 550 kilogram-meters of work per minute for 30 minutes was followed by urinary excretion of creatine in 5 out of 8 experiments in which 500 cc. water were ingested immediately before the exercise.

Creatine appeared in the urine in 17 out of 19 experiments in which exercise was preceded by the ingestion of 50 grams glucose, 50 grams fructose or a mixture of 25 grams glucose and 25 grams fructose.

In the exercise, as in the rest experiments, more creatine was excreted following the ingestion of fructose than with glucose or a mixture of the sugars.

Creatine was excreted more frequently in the exercise experiments with the sugars than in the rest experiments with the sugars, or the exercise experiments with water.

Creatine excretion was not accompanied by a corresponding diminution in the creatinine excretion.

## REFERENCES

BACHMANN, G. AND J. HALDI. This Journal Proc. **113:** 3, 1935.
BENEDICT, S. R. J. Biol. Chem. **18:** 191, 1914.
J. Biol. Chem. **82:** 1, 1929.
CARPENTER, T. M. AND E. L. FOX. Arbeitsphysiol. **4:** 527, 570, 1931.
CATHCART, E. P. J. Physiol. **38:** 311, 1909.
DEUEL, H. J. J. Biol. Chem. **75:** 367, 1927.
FOLIN, O. J. Biol. Chem. **17:** 469, 1914.
HALDI, J. AND G. BACHMANN. This Journal Proc. **113:** 58, 1935.
HAWK, P. B. AND O. BERGEIM. Practical physiological chemistry, p. 833, 1931.
MENDEL, L. B. AND W. C. ROSE. J. Biol. Chem. **10:** 213, 1911.
ROSE, W. C. J. Biol. Chem. **26:** 331, 1916.

# THE RESPIRATORY METABOLISM OF STIMULATED FROG'S MUSCLE

C. L. GEMMILL

*From the Department of Physiology, Johns Hopkins University, School of Medicine, Baltimore, Md.*

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In 1928 Hill discussed the theoretical basis for the aerobic and anaerobic stimulation of frog's sartorius muscle. Since that time, considerable data on this preparation have accumulated. Hill and Kupalov (1929) showed that the sartorius in oxygen responded more than 10,000 times before complete fatigue and in nitrogen more than 2500 times. Gemmill, in 1932, measured the lactic acid formed during slow stimulation of the sartorius under anaerobic conditions in Ringer's solution and found that the amount of acid (to 1.2 per cent) produced was proportional to the product of the tension developed and the length of the muscle. The respiratory quotient for this muscle stimulated in oxygen averaged 0.97. Later (Gemmill, 1934) lower average values were obtained; 0.90 by using a manometric method and 0.94 with a volumetric apparatus. In this paper the literature on respiratory quotients of resting and stimulated muscles was reviewed. In 1935 Gemmill compared the oxygen consumption with the utilization of total fermentable carbohydrate in sartorius muscle by stimulating the muscles in oxygenated Ringer's solution nine times a minute for periods of three to six hours. It was discovered that the utilization of carbohydrate accounted for only part (average 42 per cent) of the total energy used as determined from the oxygen consumption.

Newer methods for the determinations of respiratory metabolism of isolated tissues than those used in the previous work have been developed by Dickens and Šimer (1933) and by Dixon and Keilin (1933). These authors have described apparatus for the measurement of respiratory quotients in bicarbonate buffered solutions. For the present work the method of Dixon and Keilin (1933) was chosen because it has certain advantages over the older methods. For example, a separate manometer for determining preformed carbon dioxide is unnecessary. Furthermore, a qualitative estimate of the respiratory quotient may be obtained without elaborate calculations since the position of the fluid in the two limbs of the manometer, after the acid has been spilled over the stimulated muscle, gives a rough estimate of this quotient.

METHODS. A vessel of the Dixon-Keilin type was modified by the addition of a side tube (fig. 1 *B*) through which a muscle lever (Meyerhof, Möhle and Schulz, 1932) was passed and held firmly in place by springs. The tension developed by the muscle was recorded by focusing a beam of light through an optically flat lens, *A*, on the mirror of the muscle lever. Stopcocks were placed between the manometer and the vessels. These stopcocks were closed during the period of stimulation. This procedure permitted the use of manometer fluid of specific gravity of 1.034. Therefore, greater changes in the readings of the manometer were obtained than would have been given with the Clerici fluid used by Dixon and Keilin (1933). Dickens and Greville (1933) used stopcocks in this position in their constant volume differential manometer.

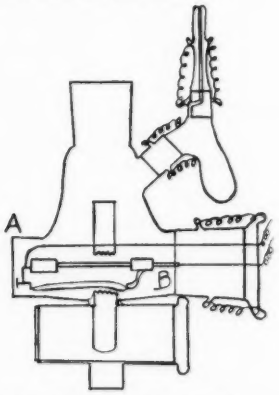


Fig. 1. Diagram of modification of Dixon-Keilin vessel for determination of respiratory quotients of stimulated muscles in bicarbonate buffered solutions.

The Ringer's solution was made according to the directions given by Barkan, Broemser and Hahn (1922). Before being placed in the vessels, this solution was saturated with 2.5 per cent carbon dioxide in oxygen at the temperature of the water bath. In addition, the gas mixture was passed through the vessels after they were placed in the water bath. After ten minutes of shaking, the tubes were disconnected and the vessels were shaken for an additional five minutes in order that the two sides might be equilibrated at atmospheric pressure. The gas had been saturated previously with water vapor at the temperature of the bath before it was passed over the solutions in the vessels (Gemmill, 1934). The rate of shaking was 136 times a minute. The temperature of the bath for the series of experiments made in May was 16.1°C. ± 0.01. For the experiments in November, the bath temperature was 16.6° ± 0.01. Two different sets of vessels and manometers were used. The volume of one of the

vessels in the set used in May was 48.265 cc. and the volume of a vessel from the other set was 33.432 cc. The rest of the procedure was the same as that described by Dixon and Keilin (1933). The muscles were stimulated with maximal make induction shocks at a rate of nine to ten times a minute.

RESULTS. The results are given in table 1 and figure 2. The respiratory quotient averaged 0.90 with variations from 0.86 to 0.98. The ratio between the oxygen consumption and the product of tension and length ($Km_{O_2}$) was higher than in the former series. The average value was 1430 in this series in contrast to 1300 obtained in the former group (Gemmill, 1934). The average tension developed by these muscles and

TABLE 1

*Summary of experiments*

| DATE | NUMBER OF MUSCLES | WEIGHT | | LENGTH | $CO_2$ PER GRAM (WET) HOUR | $O_2$ PER GRAM (WET) HOUR | R.Q. | $Q_{O_2}$ | TENSION PER GRAM (WET) HOUR | $Km_{O_2}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Wet | Dry | | | | | | | |
| | | *gram* | *gram* | *cm.* | *mm.*$^3$ | *mm.*$^3$ | | | *kgm.* | |
| May 6 | 1 | 0.092 | 0.0159 | 4.3 | 508 | 554 | 0.92 | 3.2 | 347 | 1,880 |
| May 9 | 2 | 0.138 | 0.0215 | 4.1 | 278 | 308 | 0.90 | 2.0 | 154 | 1,430 |
| May 10 | 1 | 0.075 | 0.0085 | 4.0 | 137 | 154 | 0.89 | 1.4 | | |
| May 11 | 2 | 0.156 | 0.0230 | 4.0 | 409 | 449 | 0.91 | 3.0 | 201 | 1,260 |
| May 14 | 2 | 0.156 | 0.0225 | 4.0 | 268 | 300 | 0.89 | 2.2 | 112 | 1,050 |
| May 15 | 2 | 0.185 | 0.0313 | 4.0 | 316 | 366 | 0.86 | 2.2 | 159 | 1,220 |
| Nov. 5 | 1 | 0.146 | 0.0258 | 3.8 | 269 | 295 | 0.91 | 1.7 | 107 | 970 |
| Nov. 6 | 1 | 0.187 | 0.0353 | 4.0 | 358 | 367 | 0.98 | 1.9 | 238 | 1,820 |
| Nov. 8 | 1 | 0.171 | 0.0314 | 3.9 | 316 | 365 | 0.87 | 2.0 | 198 | 1,480 |
| Nov. 11 | 1 | 0.174 | 0.0349 | 4.0 | 372 | 395 | 0.95 | 2.0 | 219 | 1,550 |
| Nov. 12 | 1 | 0.140 | 0.0250 | 3.9 | 314 | 358 | 0.88 | 2.0 | 248 | 1,890 |
| Nov. 13 | 1 | 0.172 | 0.0328 | 3.9 | 333 | 376 | 0.88 | 2.0 | 167 | 1,220 |
| Averages | | | | | | 357 | 0.90 | 2.1 | 195 | 1,430 |

the oxygen consumed were less than the values obtained in the former series with the muscles stimulated at the same rate.

DISCUSSION. The isolated sartorius muscle of the frog stimulated under aerobic conditions has been shown to have a respiratory quotient of less than unity by three different methods, a manometric method using phosphate buffered solution, a volumetric (Thunberg) method (Gemmill, 1934) and the Dixon-Keilin method using a bicarbonate buffered medium.

The volumetric method and the Dixon-Keilin method have the advantage of giving a qualitative estimation of the respiratory quotient without calculation. In the present method, after the acid had been poured over the stimulated muscle, the fluid in the manometer was always higher on this side than on the control side. Therefore, the carbon dioxide given

off by the muscle must have been less than the oxygen taken in during the stimulation. Actual calculations showed that the quotient had an average value of 0.90. This fact corroborates the former observation (Gemmill, 1935) that carbohydrate supplies only part of the energy for long continued muscular contraction.

The oxygen consumption of the present series averaged 357 mm.$^3$ per gram per hour and the average tension was 195 kgm. per gram of muscle per hour. These values are lower than those obtained for the sartorius muscle stimulated at the same rate in a former series (Gemmill, 1935).

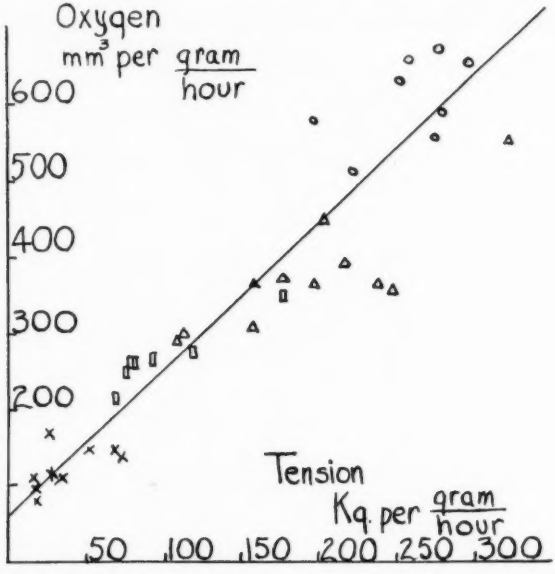


Fig. 2. Graphic relationship of oxygen consumed and tension developed for stimulated sartorii. × Gemmill, 1932, □ Gemmill, 1934, ○ Gemmill, 1935, △ Gemmill, present results.

The initial length of the muscles in the former group was slightly greater than in this series. This factor does increase the oxygen consumed by contracting muscles (Meyerhof, Gemmill, Benetato, 1932). The time of the year and the differences in the two media also may have played a rôle in this difference.

A graphic summary of the relation of the oxygen consumption to tension developed of all the experiments in which the sartorius muscle has been stimulated under aerobic conditions is given in figure 2. The different sets of values were obtained by varying the rates of stimulation. A

straight line relationship holds throughout the range of the experiments. The line crosses the ordinate for zero tension at 30 mm.$^3$ of oxygen, a value corresponding to the resting oxygen consumption of this preparation (Gemmill, 1934).

SUMMARY

The respiratory metabolism of the stimulated frog's sartorius muscle was determined by a modification of the Dixon-Keilin method. The respiratory quotient averaged 0.90 with variations from 0.86 to 0.98 in the bicarbonate buffered solutions. The oxygen consumed per gram of muscle averaged 357 mm.$^3$ and the tension developed gave an average value of 195 kilograms per gram of muscle per hour.

A graphic summary is given of the oxygen consumed and the tension developed in thirty-five experiments for the frog's sartorius stimulated under aerobic conditions. A straight line relationship holds throughout the range of these experiments.

REFERENCES

BARKAN, G., P. BROEMSER AND A. HAHN. Ztschr. f. Biol. **74:** 1, 1922.
DICKENS, F. AND F. ŠIMER. Abderhalden's Handbuch. Abt. IV, Teil **13:** 435, 1933.
DICKENS, F. AND G. GREVILLE. Biochem. J. **27:** 1479, 1933.
DIXON, M. AND D. KEILIN. Biochem. J. **27:** 86, 1933.
GEMMILL, C. L. Biochem. Ztschr. **246:** 319, 1932.
J. Cell. and Comp. Physiol. **5:** 277, 1934.
This Journal **112:** 294, 1935.
HILL, A. V. Proc. Roy. Soc. B. **104:** 39, 1928.
HILL, A. V. AND P. KUPALOV. Proc. Roy. Soc. B. **105:** 313, 1929.
MEYERHOF, O., W. MÖHLE AND W. SCHULZ. Biochem. Ztschr. **246:** 285, 1932.
MEYERHOF, O., C. L. GEMMILL AND G. BENETATO. Biochem. Ztschr. **258:** 371, 1933.

# THE EFFECT OF PROGESTIN ON THE IN VITRO RESPONSE OF THE RABBIT'S UTERUS TO PITUITRIN

A. WATTS MAKEPEACE, GEORGE W. CORNER AND WILLARD M. ALLEN

*From the Departments of Anatomy and of Obstetrics and Gynecology, The University of Rochester, School of Medicine and Dentistry*

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In a series of papers by Knaus (1929, 1930a), dealing with the pharmacodynamics of the uterus, it was established that the excised rabbit's uterus is refractory to pituitrin in vitro when it is under the influence of a functioning corpus luteum. Thus, during either pregnancy or pseudopregnancy the addition of pituitrin to the bath in which the excised uterus was suspended failed to cause any response, whereas the addition of a similar amount of pituitrin brought about a prompt contraction when the uterus from an oestrous rabbit was used. These results led Knaus (1930b) to inject sexually mature, unmated rabbits with corpus luteum extracts prepared by the Corner-Allen method and known to contain progestin, to find out whether or not such extracts would cause inhibition to pituitrin in the same manner as the rabbit's own corpora lutea. The results were as expected; uteri under the influence of corpus luteum extracts failed to respond to pituitrin. The preparations used were quite crude and consequently the results obtained did not indicate that the pituitrin-inhibiting factor was necessarily the same as the one responsible for endometrial proliferation (progestin).

More recently Robson and Illingworth (1931) and de Fremery, Luchs and Tausk (1932) have studied more extensively various fractions obtained in the purification of corpus luteum extracts and have obtained an apparent separation of two active principles, one causing progestational proliferation of the endometrium (progestin), and the other bringing about desensitization to pituitrin (desensin). Fractions that induced proliferation could be prepared which failed to produce pituitrin desensitization and vice versa, but a complete separation of the two supposed principles was not obtained; and moreover, when the same fractions were studied in vivo (Tausk, de Fremery, Luchs and Reynolds, 1934), desensitization to pitocin was only apparent when the progestin-containing fraction was injected. Foster (1933) has also reported that there is a gradual loss of pituitrin-inhibiting substance as the extracts are increasingly purified and that uteri treated with crystalline material show a

maximal progestational modification and yet give a maximal pituitrin response.

The above experiments were carried out on excised uteri in vitro, the activity of the corpus luteum or of the corpus luteum extract being measured by the reaction to pituitrin added directly to the bath. A somewhat more natural means of studying the effect of hormones on uterine motility has been devised by Reynolds (1930), Reynolds and Friedman (1930), a small rubber balloon being inserted into the uterine cavity and the in vivo contractions recorded as they occur spontaneously. By use of this method it has also been shown that the uterine muscle is quiescent when under the influence of the corpus luteum and very active when under the action of oestrin (Reynolds and Friedman, 1930; Reynolds, 1931); in this type of experiment crude corpus luteum extracts were found to suppress oestrous contractions very effectively (Reynolds and Allen, 1932). Therefore, by either the in vitro method of Knaus or the in vivo method of Reynolds, the uterine muscle appears to be in a relative state of quiescence when under the influence of either the rabbit's own corpora lutea or progestin-containing extracts of the corpus luteum.

The question of what hormone was responsible for the inhibition in vivo produced by these extracts has been recently answered by experiments using progesterone (crystalline progestin) in which it was found that the pure substance possessed the same inhibiting power as crude extracts (Allen and Reynolds, 1935). It is apparent, therefore, that the induction of progestational proliferation of the endometrium and the inhibition in vivo of oestrous motility are both properties of progesterone. Whether the inhibition of pituitrin in vitro is also a property of progesterone is the subject of this paper.

The first group of experiments was carried out essentially according to the method of Knaus. Sexually mature rabbits, in which surgical exploration had shown no corpora lutea, were injected with varying amounts of crude progestin in 4 doses at 12 hour intervals, and then autopsied about 14 hours after the last injection. One cornu was removed and suspended in 250 cc. of oxygenated Ringer-Locke solution kept at 37°C. After about one-half hour or less regular contractions would begin and then, when a satisfactory record of the spontaneous contractions was obtained, 12.5 international units of pituitrin were added to the bath. In several other cases sexually mature rabbits were castrated 14 to 18 hours after mating and then given progestin in 4 injections twice daily, thereby approximating the experiments of Robson and Illingworth.

The results from these studies (table 1) show that inhibition to 12.5 international units of pituitrin was obtained in many instances and practically complete inhibition was produced even with crystalline material and with doses comparable to those used by Knaus, but for some reason

other animals showed no inhibition whatsoever. It is also apparent that the animals in which the ovaries were not removed were better inhibited than those castrated prior to the injections; in fact complete inhibition was not obtained, in any animal in which the ovaries were removed, with doses of from 1 to 6 rabbit units. Just what the explanation of this apparent need for the ovaries may be is still uncertain, but it

TABLE 1

*Comparison of effect of pituitrin in vitro on uteri from castrated and non-castrated rabbits, treated with progestin for 2 days*

| RABBIT NUMBER | EXTRACT NUMBER | SOLIDS | PROGESTIN | PITUITRIN | RESULT |
|---|---|---|---|---|---|
| Ovaries removed 18 hours after mating | | | | | |
| | | *grams* | *Rb. U.* | *Int. U.* | |
| 1 | 302 | 3.0 | 6.0 | 12.5 | ++ |
| 2 | 302 | 1.5 | 3.0 | 12.5 | + |
| 3 | 302 | 0.8 | 1.6 | 12.5 | + |
| 4 | 301 | 0.75 | 1.5 | 12.5 | ++ |
| 5 | 301 | 0.75 | 1.5 | 12.5 | + |
| 6 | 301 | 0.75 | 1.5 | 12.5 | + |
| 7 | 301 | 0.5 | 1.0 | 12.5 | ++ |
| 8 | 302 | 0.75 | 1.5 | 12.5 | ++ |
| 9 | 302 | 0.5 | 1.0 | 12.5 | ++ |
| 10 | 301 | 0.5 | 1.0 | 12.5 | ++ |
| Unmated animals, ovaries intact | | | | | |
| 11 | 301 | 1.0 | 2.0 | 12.5 | − |
| 12 | 302 | 0.8 | 1.6 | 12.5 | ± |
| 13 | 301 | 0.5 | 1.0 | 12.5 | − |
| 14 | 301 | 0.5 | 1.0 | 12.5 | ± |
| 15 | 103 K | 0.6 | 1.5 | 12.5 | + |
| 16 | $\alpha$ progesterone | 0.0017 | 1.7 | 12.5 | ± |
| 17 | $\alpha$ progesterone | 0.0013 | 1.3 | 12.5 | + |
| 18 | $\beta$ progesterone | 0.0019 | 1.9 | 12.5 | ± |

is of no importance in demonstrating the inhibitory rôle of progestin, since experiments to be described subsequently show that good pituitrin inhibition can be obtained under the proper experimental conditions in castrated animals. It is of importance to note, however, that fairly good inhibition was obtained in one case with 1.7 mgm.[1] of $\alpha$ progesterone and

[1] At a meeting of the Biological Standards Commission of the League of Nations held in London, July 15–17, 1935, dealing with international standards for the sex hormones, it was agreed that 1 international unit of progesterone should be defined as the progestational activity of 1 mgm. of $\beta$ progesterone. Therefore, 1 Corner-Allen rabbit unit is equivalent to 1 international unit, and for practical purposes the units used in this paper are equivalent to international units, although the international standard is not yet available.

in another instance with 1.9 mgm. of $\beta$ progesterone. A positive response was obtained with 1.3 mgm. of $\alpha$ progesterone.

The lack of definite inhibition in other experiments of the above series was conceivably due to either the large quantity of pituitrin used (12.5 int. u.) or to inadequate dosage of progestin. Consequently a second group of experiments was carried out in which sexually mature rabbits castrated 14 to 18 hours after mating were injected four times at 12 hourly intervals with varying amounts of progestin ranging from 0.1 rb. u. to 12 rb. u. The reaction of the uterus was studied as in the above group except that 1 int. u. of pituitrin was used rather than 12.5. The results of this study are recorded in table 2 from which it is apparent that as the amount of progestin was increased the number of animals showing complete (0) or almost complete (±) inhibition increased. Of those receiving 2 rb. u. or more, 62.5 per cent showed complete inhibition, 12.5 per cent partial inhibition and 25 per cent no inhibition. In contrast, of those getting 1.0 to 2.0 rb. u. only 31 per cent showed complete inhibition, and in the group receiving less than 1.0 rb. u. there was none showing complete inhibition. These findings demonstrated that a considerable variation may be expected in the assay of pure materials by this method but, to be more certain of that, 11 rabbits—7 castrated and 4 not castrated—were each injected with 1.5 mgm. of $\beta$ progesterone. In this group, 2 (18 per cent) showed complete inhibition, 4 (36 per cent) partial inhibition, and 5 (46 per cent) no inhibition. It was quite evident, therefore, that if uniform results were to be obtained the experimental procedure would have to be modified somewhat.

TABLE 2

*Effect of pituitrin in vitro on uteri from rabbits injected for 2 days with progestin*

| NUMBER OF ANIMALS | TOTAL SOLIDS | TOTAL PROGESTIN | RESPONSE TO 1 UNIT PITUITRIN | | |
|---|---|---|---|---|---|
| | | | 0 | ± | + and ++ |
| Partially purified progestin | | | | | |
| | *mgm.* | *Rb. U.* | *per cent* | *per cent* | *per cent* |
| 16 | 6 to 1320 | 2 to 12 | 62.5 | 12.5 | 25 |
| 16 | 4 to 132 | 1 to 2 | 31.2 | 18.8 | 50 |
| 7 | 9 to 65 | <1 | 0 | 14.3 | 85.7 |
| $\beta$ progesterone | | | | | |
| 11* | 1.5 | 1.5 | 18.2 | 36.4 | 45.4 |

* Four animals in this group were not castrated. All others were castrated approximately 18 hours after mating.

The general impression from these studies, carried out in animals in-

jected for only 2 days, was that progesterone was as effective in causing inhibition to pituitrin as were any of our impure preparations; but with neither the pure nor impure preparations could consistent results be obtained under presumably the same conditions. This irregularity could be due to unavoidable variability of animals but at the same time made it highly desirable to find if possible other conditions under which more consistent results could be obtained. An indication that a longer period of injection might be beneficial presented itself when the uteri removed from the above animals injected for only 2 days were studied histologically. In general the proliferative changes observed in these bore no direct relation whatsoever to dose, the several animals each receiving 1.5 mgm. of $\beta$ progesterone showing wide variation in the degree of proliferative changes present. This is in direct contrast to animals injected with progesterone for 5 days according to the Corner-Allen method, in which over the proper dosage range the degree of proliferation follows very closely the amount of hormone injected. The cause for the irregularity in response when the shorter period of injection is used may be and probably is due to irregularity of absorption. Consequently we were led to try a longer injection period.

For this study sexually mature rabbits were castrated 18 to 20 hours after mating, only those in which ovulation occurred being used, and then injected 2 times daily at approximately 12 hour intervals for 5 days with the desired amount of extract dissolved in 1 cc. total of sesame oil. Autopsy was carried out from 12 to 14 hours after the last injection and one cornu studied in vitro as in the other groups. Several preparations of $\beta$ progesterone were compared with similar physiological doses of a highly purified oily preparation of which 4.0 mgm. contained 1 rb. u. This type of experiment enabled us for the first time to get consistent results. When either 1 rb. u. per day of the impure extract or 1 mgm. (= 1 rb. u.) of $\beta$ progesterone was given, complete inhibition was obtained in every case (table 3). With doses smaller than this, variable results were noted but the majority of animals getting more than 0.2 rb. u. per day showed partial inhibition, while the majority getting 0.2 rb. u. or less showed little or no inhibition.

These results demonstrate that complete inhibition to pituitrin in vitro can be obtained with the progesterone isolated from pigs' ovaries providing an adequate amount of material is given for a sufficiently long time, and that rabbit unit for rabbit unit, the pure preparation behaves in the same manner as a partially purified one. If these findings mean that both physiological responses, proliferation of the endometrium and inhibition to pituitrin, are due to the same substance, then synthetic progesterone prepared from stigmasterol should have both of these physiological properties. Consequently experiments with the synthetic preparation were carried out.

The synthetic progesterone used was prepared from stigmasterol according to the method of Butenandt (1934) and was supplied in a partially purified state by Doctor Schwenk of the Schering Corporation. This

TABLE 3

*Effect of pituitrin in vitro on uteri from castrated rabbits injected for 5 days with progestin*

| NUMBER OF ANIMALS | TOTAL SOLIDS | TOTAL PROGESTIN | RESPONSE TO 1 UNIT PITUITRIN | | | |
|---|---|---|---|---|---|---|
| | | | 0 | ± | + | ++ |
| **Purified progestin** | | | | | | |
| | *mgm.* | *Rb. U.* | *per cent* | *per cent* | *per cent* | *per cent* |
| 4 | 20 | 5 | 100 | 0 | 0 | 0 |
| 6 | 12 | 3 | 33.3 | 50 | 16.7 | 0 |
| 5 | 8 | 2 | 20 | 40 | 20 | 20 |
| 1 | 4 | 1 | 0 | 0 | 100 | 0 |
| 4 | * | 0 | 0 | 0 | 0 | 100 |
| **β progesterone from pigs' ovaries** | | | | | | |
| 4 | 5 | 5 | 100 | 0 | 0 | 0 |
| 2 | 4 | 4 | 50 | 50 | 0 | 0 |
| 2 | 3 | 3 | 0 | 100 | 0 | 0 |
| 2 | 2 | 2 | 0 | 0 | 100 | 0 |
| 6 | 1 | 1 | 0 | 33.3 | 66.7 | 0 |
| 3 | 0.5 | 0.5 | | 33.3 | 33.3 | 33.3 |
| **β progesterone from stigmasterol** | | | | | | |
| 2 | 5.0 | 5 | 100 | 0 | 0 | 0 |
| 2 | 1.0 | 1 | 50 | 0 | 50 | 0 |
| 2 | 0.5 | 0.5 | 0 | 0 | 50 | 50 |
| **Summarized results** | | | | | | |
| 10 | | 5 | 100 | 0 | 0 | 0 |
| 2 | | 4 | 50 | 50 | 0 | 0 |
| 8 | | 3 | 25 | 62.5 | 12.5 | 0 |
| 7 | | 2 | 14.3 | 28.5 | 42.9 | 14.3 |
| 9 | | 1 | 11.1 | 22.2 | 66.7 | 0 |
| 5 | | 0.5 | 0 | 20 | 40 | 40 |
| 4 | | 0 | 0 | 0 | 0 | 100 |

* Controls consist of 1 animal each given mazola, wheat germ oil, 12.6 mgm. pregnandiol and 20 mgm. of allo-pregnanolon.

preparation was subjected to fractional crystallization from dilute methyl alcohol, a yield of 50 mgm. of β progesterone being obtained from 75 mgm. of impure crystals. The crystals prepared had the same form as those isolated from pigs' ovaries. They were injected in varying doses for

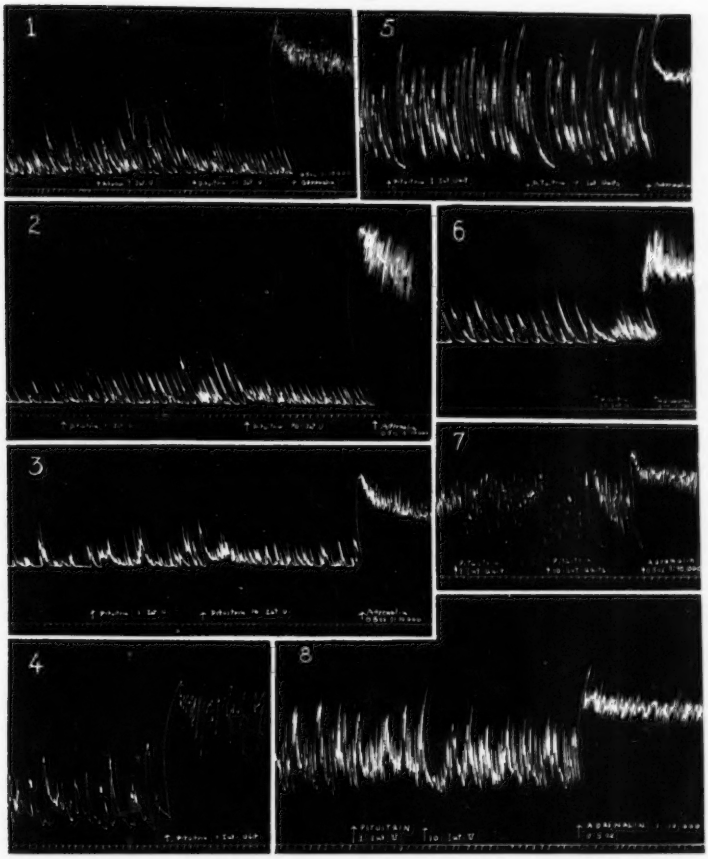
5 days in the same manner as in the above experiments of the 5 day type. Two animals (table 3), each receiving 1 mgm. per day, showed no response whatsoever to pituitrin; of two animals each receiving 0.2 mgm. per day, one showed a + response and the other a 0 response, and of the two receiving 0.1 mgm. per day one showed a + and the other a ++ response. These results show that the synthetic material has the same capacity to inhibit pituitrin in vitro as has the compound isolated from pigs' ovaries.

The criteria followed in the appraisal of the pituitrin response in all experiments have been very stringent since those tabulated as completely negative showed absolutely no detectable change in the type of spontaneous contractions other than an occasional transient relaxation (figs. 1, 5, 7, 8). Those designated as ± showed only a slight increase in the frequency of contractions with no change in tonus (figs. 2, 6). In many cases these effects are so slight as to make the result almost negative. A + response was one in which there was a more marked increase in tonus (fig. 3) but still much less than the typical pituitrin response found in the oestrous animal or the animal castrated either for two or five days. In these conditions 1 int. u. of pituitrin produces a pronounced increase in tonus; such responses as that have been classified as ++ (fig. 4). The responses recorded in the tables apply only to the first dose of pituitrin added to the bath. In many instances a second, larger dose (10 int. u.) was given but this failed in most instances to induce a contraction when the first dose failed. However, frequently the larger dose did inhibit the contractions still more than the first dose in uteri which failed to show the typical pituitrin response.

The crystalline preparations used have in every case not been absolutely pure in that the melting points were not 120.5° to 121° for the $\beta$ form or 128° for the $\alpha$ form. All those designated as $\beta$ progesterone, both natural and synthetic, were recrystallized at least two times after the typical needle form was apparent and no preparation had a melting point below 117°. The one preparation of $\alpha$ progesterone used melted at 127° and was of the typical prism form. In no case did the slight amount of impurity responsible for this negligible depression of the melting point have any noticeable effect on the proliferating capacity. The progesterone used was prepared from pig ovaries by a new method soon to be published (Allen and Goetsch). The purity of the other extracts used is indicated also in the tables by the amount of solids injected.

The crystalline preparations of natural origin used in the 5 day experiments produced proliferation in all cases, but it is worthy of note that a +++[2] proliferation was produced with only 0.5 mgm. in 2 out of 3 cases and that full proliferation was produced in every case with 1.0 mgm.

[2] For illustrations showing the varying degrees of proliferation to which the + signs refer, see W. M. Allen (1930).




Figs. 1, 2, 3 and 4 show the effects of pituitrin in vitro following administration of 5 mgm., 3 mgm. and 2 mgm. of $\beta$ progesterone, and 1 cc. Mazola (control) respectively for the first 5 days after castration and show 0, $\pm$, + and ++ responses. These are representative of the types of response recorded in the tables.

Fig. 5 shows a 0 response to both 1 and 10 units of pituitrin following administration of 5.0 Rb. U. of impure progestin for the first 5 days after castration. Note that there is a transient relaxation after both 1 and 10 units of pituitrin.

Fig. 6 shows a $\pm$ response from 12.5 units of pituitrin following administration of 1.7 mgm. of $\alpha$ progesterone in 2 days. Ovaries intact.

Fig. 7 shows a 0 response (except for relaxation) to both 1 and 10 units of pituitrin following administration of 1.5 mgm. of $\beta$ progesterone in the first 2 days after castration.

Fig. 8 shows a 0 pituitrin response to 1 unit and transient relaxation to 10 units following administration of 5.0 mgm. of synthetic $\beta$ progesterone during the first five days after castration.

The animals receiving synthetic progesterone also showed a similar response. Each of the animals getting 1.0 mgm. showed a ++++ proliferation; of those getting 0.5 mgm. each, one showed a +++ proliferation and the other + proliferation. These preparations, therefore, produced somewhat better proliferation than those previously reported (Wintersteiner and Allen, 1934), but the two studies are not exactly comparable because the animals used in this present group were injected twice daily whereas those reported in the earlier paper were injected only once daily.

Discussion. The experiments described above, designed to compare the effect of progesterone, both natural and synthetic, with impure corpus luteum extracts containing the hormone on the response of the uterus to pituitrin in vitro, show that under the proper experimental conditions the pure substance brings about complete desensitization to pituitrin and that rabbit unit for rabbit unit (as measured by proliferation), it produces the same effect as a partially purified extract. When it is remembered that the potencies of both the crude and the pure preparations are originally determined by an entirely different biological test, i.e., by their ability to produce progestational proliferation of the endometrium, it is all the more apparent that both physiological effects, pituitrin-desensitization and endometrial proliferation, can be produced by the same substance regardless of whether it is isolated from pigs' ovaries or prepared synthetically. These findings are in agreement with those of Allen and Reynolds (1935) showing that in vivo both crude progestin and natural progesterone are equally effective in inhibiting the oestrous type of uterine contractions. Both forms of the crystals were not extensively investigated in the present experiments because of the comparatively large amount of progesterone necessary. It is very tedious and somewhat wasteful to prepare both forms of crystals but if the conditions are kept optimum fairly good yields of the needles ($\beta$ form, m.p. 120.5°–121° when pure) can be obtained. For this reason we have been content to study for the most part only the one form. In this connection, however, in one of the two-day experiments almost complete ($\pm$) inhibition was obtained with 1.7 mgm. of the $\alpha$ form.

The results obtained by Robson and Illingworth (1931) and de Fremery, Luchs and Tausk (1932) in which a partial separation of the pituitrin-desensitizing substance appeared to be obtained, are not explained by these experiments. However, when the marked variation in results with a constant dosage is considered, as in the experiments recorded in table 2, it is apparent that a large number of animals would be necessary for an accurate bioassay of any two fractions. It is possible, of course, that these workers may have separated another compound which produces desensitization to pituitrin but which fails to produce endometrial proliferation. Such a compound presumably would be closely related to

progesterone and, in fact, inactive substances are found in the urine of pregnant women (pregnandiol) and in corpus luteum extracts (allo-pregnanolon) which are closely related to progesterone. However, we have injected 20 mgm. of the oxyketone (allo-pregnanolon), isolated along with the progesterone, in one experiment and 12 mgm. of pregnandiol in another but neither showed any pituitrin-inhibiting effect.

## CONCLUSIONS

Progesterone (crystalline progestin) prepared either from pigs' ovaries or synthetically from stigmasterol brings about inhibition of the in vitro reaction of the rabbit's uterus to pituitrin. This effect is produced by amounts of the same potency (as measured by proliferation) as those of impure extracts necessary to produce this effect. Consequently endometrial proliferation and pituitrin desensitization in the rabbit are both physiological properties of progesterone.

## REFERENCES

ALLEN, W. M. This Journal **92:** 174, 1930.
ALLEN, W. M. AND S. R. M. REYNOLDS. Am. J. Obstet. and Gynec. **30:** 309, 1935.
BUTENANDT, A. AND U. WESTPHAL. Ber. der deutsch. chem. Gesell. **67:** 2085, 1934.
DE FREMERY, P., A. LUCHS AND M. TAUSK. Pflüger's Arch. **231:** 341, 1932.
FOSTER, M. A. Anat. Rec. **55:** no. 4 suppl., 56, 1933.
KNAUS, H. Arch. f. Gynäk. **138:** 201, 1929.
Arch. f. Gynäk. **140:** 181; **141:** 374, 395, 1930a.
Arch. f. Exp. Path. u. Pharmakol. **151:** 371, 1930b.
REYNOLDS, S. R. M. This Journal **92:** 420, 1930.
This Journal **97:** 706, 1931.
REYNOLDS, S. R. M. AND M. H. FRIEDMAN. This Journal **94:** 696, 1930.
REYNOLDS, S. R. M. AND W. M. ALLEN. This Journal **102:** 39, 1932.
ROBSON, J. M. AND R. E. ILLINGWORTH. J. Exper. Physiol. **21:** 93, 1931.
TAUSK, M., P. DE FREMERY, A. LUCHS AND S. R. M. REYNOLDS. Acta Brevia Neerl. **4:** 85, 1934.
WINTERSTEINER, O. AND W. M. ALLEN. J. Biol. Chem. **107:** 321, 1934.

# THE GASTRIC SECRETAGOGIC VALUE OF VARIOUS DIGESTIVE SECRETIONS

M. S. KIM AND A. C. IVY

*From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago*

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It is known that the oral administration of saliva and bile stimulate gastric secretion more than water when taken in equal volumes (1, 2, 3). It is not known whether these secretions, including pancreatic juice, stimulate gastric secretion by local contact with the gastric mucosa. Babkin (1) has observed that when duodenal juices are regurgitated into the stomach their secretory effect depends on the time they are permitted to remain there, which suggests that they exert a local "secretagogue-like" action. Yet, bile is known to stimulate gastric secretion slightly when introduced into a Thiry fistula of the jejunum (3).

To ascertain with certainty if saliva, bile and pancreatic juice may stimulate gastric secretion, or exert a local secretagogue action, when in contact with the gastric mucosa, three dogs with a pouch of the entire stomach and a duodeno-esophageal anastomosis were used (4, 5). After collecting the continuous secretion of the pouch for several half-hour periods, the stomach pouch was *perfused* with 50 cc. quantities of the digestive secretion for one-half hour. The saliva was used undiluted, but the pancreatic juice and hepatic bile were diluted from one to four times by volume. The "perfusate" was titrated before and after perfusion and the secretion of the pouch was collected for four half-hour periods thereafter (for details see reference 5). The bile and pancreatic juice used in these tests was collected from animals having a "chronic" biliary or pancreatic fistula.

Typical results with saliva and bile are shown in table 1. The results with pancreatic juice were practically negative in so far as acid output is concerned. When saliva was used, the volume of the perfusing fluid was not changed, the secretory rate being equal to the rate of absorption of water from the saliva. The secretagogic effect was very slight when compared to a food such as meat juice or liver. When bile and pancreatic juice were used, the volume of the perfusing fluid was uniformly increased (from 4 to 10 cc.), meaning a slightly increased rate of secretion of gastric fluids. Free acid appeared in only one of nine experiments. The acid output during and after the perfusion with bile was augmented in every test,

but only slightly; with pancreatic juice the augmentation of acid output was so slight as to be insignificant.

It is probable that if these secretions were held in contact with the stomach longer than a half-hour, as in these experiments, the stimulation of acid output and the secretion of diluting fluid may have been greater. We did not perform such a test because under normal gastric conditions it is believed that regurgitated fluid would be evacuated from the stomach in

TABLE 1

*Assay of "gastric secretagogue" value of saliva and hepatic bile*

| PROCEDURE | TIME, 30 MINUTES | GASTRIC SECRETION | | | |
|---|---|---|---|---|---|
| | | Volume | Free acid | Total acid | Output HCl |
| | | cc. | | | mgm. |
| Control | 1 | 2.2 | 0 | 0.027 | 0.60 |
| | 2 | 1.8 | 0 | 0.027 | 0.50 |
| | 3 | 4.4 | 0 | 0.027 | 1.20 |
| Perfused pouch with 50 cc. of saliva | | | | | |
| During perfusion | 30 minutes | No increase | | | 4.6 |
| After perfusion | 1 | 5.8 | | 0.03 | 1.6 |
| | 2 | 2.1 | 0.06 | 0.11 | 2.3 |
| | 3 | 2.4 | 0.11 | 0.15 | 3.7 |
| | 4 | 2.8 | 0.06 | 0.13 | 3.6 |
| Control | 1 | 1.6 | 0 | 0.01 | 0.2 |
| | 2 | 2.0 | 0 | 0.01 | 0.27 |
| | 3 | 2.5 | 0 | 0.01 | 0.34 |
| Perfused pouch with hepatic bile | | | | | |
| During perfusion | 30 minutes | Increase 8.0 | | | 11.2 |
| | 1 | 3.6 | 0 | 0.05 | 1.9 |
| | 2 | 1.2 | 0 | 0.07 | 0.8 |
| | 3 | 0.8 | 0 | 0.05 | 0.4 |
| | 4 | 0.5 | 0 | 0.02 | 0.1 |

a half-hour, and we were primarily interested in ascertaining the "normal" gastric secretagogic value of these digestive secretions.

## CONCLUSION

Thus, saliva and hepatic bile when in contact with the gastric mucosa stimulate secretion by a local secretagogic action only very slightly, the latter more than the former; pancreatic juice has only a very slight local

secretagogic action if any. Bile and pancreatic juice stimulate the production of a mucoid or diluting fluid by the gastric mucosa to a slight extent in the dilutions used.

## REFERENCES

(1) Babkin. Emanuel Libman Anniversary Volume, 1932, Internat. Press, N. Y.
(2) Ivy. This Journal **46:** 420, 1918.
(3) Meyer, Ivy and McEnery. Arch. Int. Med. **34:** 129, 1924.
(4) Lim, Ivy and McCarthy. Quart. J. Exper. Physiol. **15:** 13, 1925
(5) Kim and Ivy. This Journal **105:** 220, 1933.

# STUDIES IN AVIAN CARBOHYDRATE METABOLISM

RANDALL SPRAGUE AND A. C. IVY

*From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago*

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That the metabolic disturbance resulting from removal of the pancreas in birds differs essentially from that similarly produced in dogs has been known for many years. Although the observations of early workers in the field of experimental avian diabetes were not in complete agreement, it has been more recently shown (1, 2) in chickens and ducks that total removal of the pancreas causes hyperglycemia only for a relatively brief period, after which the blood sugar returns to its preoperative level, the liver stores glycogen, and the birds apparently resume their normal state, except for the digestive disorders and inanition resulting from lack of the external secretion of the pancreas.

It has been found (3) that the pancreas of normal chickens yields approximately as much "insulin" as has been reported to occur in the pancreas of calves, and that "insulin" was not extractable from the tissues of the depancreatized chicken. It has been observed (2) that the liver, kidney and muscle tissue of normal ducks yields insulin on extraction, but the same tissues from depancreatized ducks do not, and that the respiratory quotient of ducks is not markedly influenced by pancreatectomy, and (4) in depancreatized ducks is almost normally elevated by feeding glucose by stomach tube.

We have undertaken a further series of experiments on normal and depancreatized ducks in an attempt to learn more concerning the mechanisms involved in their carbohydrate metabolism.

METHODS. Young white domestic ducks weighing initially from four to five pounds were used. For operation they were anesthetized with 60 to 75 mgm. of sodium amytal per kilogram, given intraperitoneally. By careful dissection the entire pancreas was removed, especial care being taken to preserve the pancreatico-duodenal vein, since ligation of this vessel results in gangrene of the duodenum and death of the animal. No macroscopic pancreatic remnants or "rests" were found at autopsy. Blood sugar was determined by the Folin modification (5) of the Folin-Wu method, a laked blood filtrate being made by the method of Haden (6).

RESULTS. The observations of Seitz and Ivy (2) concerning the very

temporary character of extirpation diabetes in ducks were confirmed. In most cases the blood sugar reached a maximum (usually not over 200 mgm. per 100 cc.) on the first day after operation, and then declined to normal (100–125 mgm.) in the next six to eight days. In a few ducks there was little or no elevation in the blood sugar after operation. All lost weight progressively. Nine ducks which died survived pancreatectomy by 41, 53, 69, 90, 115, 118, 137, 145 and 163 days' respectively. Three ducks were killed for liver glycogen determinations at 100, 121 and 162 days, respectively, following pancreatectomy.

To determine the influence of excitement on the blood sugar, determinations were made at half-hour intervals on four normal ducks which were handled for the purpose of obtaining blood samples. The results shown in table 1 indicate that there is no significant "emotional" hyperglycemia in the duck, which confirms observations on the chicken (1).

A series of experiments was then done in which standard doses of certain physiologic substances known to influence carbohydrate metabolism

TABLE 1

*Effect of handling normal ducks*

| DUCK | CONTROL | ½ HOUR | 1 HOUR | 1½ HOURS | 2 HOURS | MAXIMUM VARIATION |
|---|---|---|---|---|---|---|
| | *mgm.* | *mgm.* | *mgm.* | *mgm.* | *mgm.* | *mgm.* |
| 10 | 109 | 108 | 101 | 95 | 98 | 14 |
| 12 | 105 | 113 | 111 | 111 | 125 | 20 |
| 13 | 102 | 107 | 103 | 102 | 98 | 9 |
| 14 | 104 | 108 | 125 | 126 | 118 | 22 |

were injected, and their effects followed as indicated by changes in the blood sugar level.

The blood sugar response to insulin was studied according to the method used by de Takats and Cuthbert (7) on dogs. Insulin in a solution of 0.5 clinical units per cubic centimeter was injected intravenously in doses of 0.1 unit per kilogram into seven normal and seven depancreatized ducks fasted for eighteen hours. Blood sugar determinations were made before, and 10, 20, 30, 60, 90 and 120 minutes after the injection. The average response of the normal and depancreatized groups was strikingly similar, as shown in figure 1. Minimum values were reached twenty to thirty minutes after the injection. The average fasting blood sugar was slightly higher in the depancreatized group, and the minimum blood sugar at one-half hour was slightly lower. The average fall in blood sugar in the normal group was 34 mgm. per 100 cc., and in the depancreatized group it was 51 mgm.

The response to epinephrine was studied in a similar manner. This

substance was injected intravenously into six normal and six depancreatized ducks in doses of 0.02, 0.04, 0.08, and 0.2 mgm. per kilogram, solutions of 1–10,000, 1–5,000, 1–2,000 and 1–1,000 concentration, respectively, being used for these doses. Only the largest of these doses was sufficient to elicit a definite hyperglycemia at ten to twenty minutes in almost every case. This was more marked in the normal group, where the average rise was 47 mgm. per 100 cc., than in the depancreatized group, where it was 22 mgm., as shown in figure 2. There was also a secondary rise in many cases, with a maximum at one and a half to two hours, the second peak often being higher than the first. This delayed hyperglycemia was a more prominent feature of the response of the normal than of the depancreatized ducks.

Several experiments of short and long duration were done to determine the blood sugar response to subcutaneous, intramuscular and intravenous injections of pituitary liquid (obstetrical and surgical). One normal and

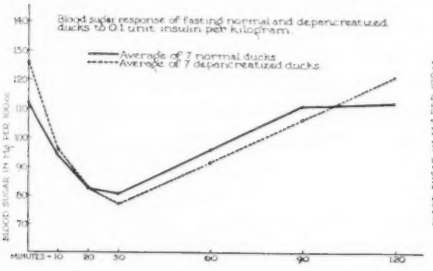


Fig. 1

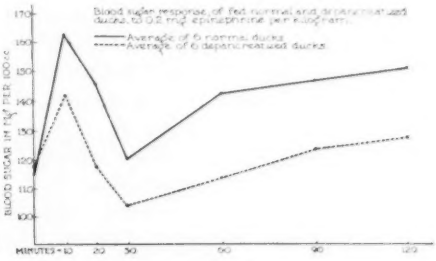


Fig. 2

one depancreatized duck showed no significant change in blood sugar for three hours following intramuscular doses of 0.5 and 0.25 cc., respectively. There was likewise no response to 0.25 cc. intravenously in a depancreatized duck. One normal and two depancreatized ducks were given 0.25 cc. subcutaneously once a day for four days, followed by the same dose twice a day for seven days. There was no notable hyperglycemia except in one of the depancreatized ducks, in which there was a moderate elevation of the blood sugar on two isolated days during the course of the treatment.

Several experiments of a somewhat different type were then performed in an attempt to discover if there is any qualitative or quantitative difference in the carbohydrate stores of normal and depancreatized ducks. Liver glycogen determinations by the Good, Kramer and Somogyi (8) modification of the Pflüger method were made on three normal and three depancreatized ducks. The birds were killed by a blow on the head and liver samples were removed at once. One of the depancreatized group (162

days) had lost much more weight than the others, and the liver glycogen content was too low to read. Excluding this duck, the depancreatized group (100 and 121 days after pancreatectomy) averaged 3.8 per cent liver glycogen, and the normal group averaged 3.9 per cent. The hydrolysate of the liver glycogen from these animals was then tested qualitatively for lactic acid. They all gave moderately strong tests, and no marked differences were noted in those from normal and depancreatized animals.

The rate of disappearance of glucose from the blood of six normal and five depancreatized ducks in vitro was studied. Sugar determinations were made immediately after the blood was drawn, and after it had stood for twenty-four hours at room temperature. In the normal group the average initial blood sugar was 116 mgm. per 100 cc., while after twenty-four hours it was 61 mgm., a decrease of 55 mgm. or 47 per cent. In the depancreatized group the average initial blood sugar was 143 mgm., while after 24 hours it was 87 mgm., a decrease of 56 mgm. or 39 per cent.

The pancreases of five normal ducks, totaling 21 grams of tissue, were extracted for insulin by the method of Fisher (9), and three quarters of the yield was injected subcutaneously into a fasting 3 kgm. rabbit. This resulted in a lowering of the blood sugar from a fasting level of 74 mgm. per 100 cc. to a minimum of 26 mgm. in two hours, indicating that an insulin-like substance is present in the pancreas of ducks. Histological sections of several duck pancreases were studied, and while no islet tissue identical with that seen in dog and human pancreas was present, there were distinct cell groups among the acini which were identified as islands. This identification was verified by Dr. L. B. Arey of the Department of Anatomy.

Discussion. As far as we know, the pancreas is the only source of insulin in the duck. If this is true, the depancreatized bird presents a unique opportunity for the study of carbohydrate metabolism which is carried on in an apparently normal manner in the absence of insulin.

The value of the method used for determining susceptibility to insulin is not definitely established. Probably blood sugar determinations are a fair measure of the activity of minute doses of insulin in experiments of short duration. We do not believe that the slight difference in the response of the normal and depancreatized groups indicates a notable difference in the factors concerned in the carbohydrate metabolism of ducks without a pancreas.

The method used for determining blood sugar response to epinephrine was essentially that of de Takats and Cuthbert (10), except for necessary alterations in the dosage. Whereas the originators of the method used a dose of 0.02 mgm. per kilogram to obtain a constant hyperglycemic response in their dogs, a dose of ten times this amount (0.2 mgm. per kilogram) was necessary in the ducks. The more marked hyperglycemic response of the normal group makes one suspect, first, that there is less

glycogen in the depancreatized duck's liver, or, second, that there is an abundance of glycogen but glycogenolysis proceeds at a slow rate. The latter explanation is supported by the finding of almost equal amounts of glycogen in the livers of the few normal and depancreatized ducks on which determinations were made. A possible fallacy in this experiment should be pointed out: The body weight of most of the depancreatized ducks was thirty to forty per cent less than their pre-operative weight. Consequently, their total dose of epinephrine was proportionately smaller than that given to the well-nourished normal ducks. It is quite possible that a comparison on the basis of a "per duck" dose rather than a "per kilogram" dose would be more just.

The dose of epinephrine which was used in ducks without ill effects is often fatal for dogs (11). It appears that epinephrine and the sympathetic nervous system play a less important rôle in avian than in mammalian carbohydrate metabolism. The evidence at hand for this view can be summarized as follows: *a*. Koppanyi et al. (1) noted no increase in blood sugar on exciting chickens. *b*. The blood sugar of ducks showed only small variations due to handling. *c*. The response of both normal and depancreatized ducks to insulin was very similar to that observed by de Takats and Cuthbert (7) in coeliac ganglionectomized dogs. (4) Relatively massive doses of epinephrine were necessary to elicit a hyperglycemic response in ducks.

Further investigation of the rôle of the pituitary and adrenals in avian carbohydrate metabolism is of course important. At the time these experiments were done, there were no reliable diabetogenic extracts of the anterior lobe available. Negative results were obtained in several experiments with preparations of unknown diabetogenic activity, e.g., "Prephysin A" (Chappel), "Antuitrin" (Parke, Davis), and several alkaline extracts made by us.

It has been postulated that following pancreatectomy in birds there is some essential change in the chemistry of their carbohydrate metabolism which enables them to dispense with insulin. The experiments performed on glycolysis of ducks' blood in vitro, on the glycogen content of the liver, and on the respiratory quotient, have yielded no evidence in support of this view.

The presence of insulin in extracts of ducks' pancreas suggests that this organ when present may play a rôle in carbohydrate metabolism similar to that of the mammalian pancreas, but its presence is not essential.

## SUMMARY

The carbohydrate metabolism of normal and depancreatized ducks was studied from several points of view. In confirmation of the work of other investigators, the slight hyperglycemia following pancreatectomy was

found to be temporary, and normal ducks exhibited no "emotional" hyperglycemia. The blood sugar response of normal and depancreatized ducks to small doses of insulin was similar. Relatively massive doses of epinephrine were necessary to elicit a hyperglycemia, and the response to a per kilogram dose was more marked in normal than in depancreatized ducks. It is suggested that epinephrine and the sympathetic nervous system probably play a less important rôle in avian than in mammalian carbohydrate metabolism. There were no significant changes in blood sugar following injections of pituitrin. Liver glycogen was present in approximately equal amounts in three normal and two depancreatized ducks, and in each case the liver glycogen hydrolysate gave a moderately strong qualitative test for lactic acid. There was no notable difference in the rate of disappearance of glucose from normal and depancreatized ducks' blood in vitro at room temperature. Ducks' pancreas yielded an insulin-like substance on extraction.

## REFERENCES

(1) KOPPANYI, T., A. C. IVY, A. L. TATUM AND F. T. JUNG. This Journal **78:** 666, 1926.
(2) SEITZ, I. AND A. C. IVY. Proc. Soc. Exper. Biol. and Med. **26:** 463, 1929.
(3) REDENBAUGH, H. E., A. C. IVY AND T. KOPPANYI. Proc. Soc. Exper. Biol. and Med. **26:** 756, 1926.
(4) SEITZ, I. AND A. C. IVY. This Journal **93:** 686, 1930.
(5) FOLIN, O. J. Biol. Chem. **67:** 357, 1926.
(6) HADEN, R. L. J. Biol. Chem. **56:** 469, 1923.
(7) DE TAKATS, G. AND F. P. CUTHBERT. This Journal **102:** 614, 1932.
(8) GOOD, C. A., H. KRAMER AND M. SOMOGYI. J. Biol. Chem. **100:** 485, 1933.
(9) FISHER, N. F. This Journal **67:** 57, 1923.
(10) DE TAKATS, G. AND F. P. CUTHBERT. Arch. Surg. **26:** 750, 1933.
(11) SOLLMANN, T. A manual of pharmacology, p. 436, Philadelphia, 1926.

# TEMPERATURE CONDITIONS IN THE BONE MARROW OF RABBIT, PIGEON AND ALBINO RAT[1]

CHARLES HUGGINS, B. H. BLOCKSOM, JR. AND W. J. NOONAN

*From the Department of Surgery of The University of Chicago*

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This report is presented since no data on the thermal conditions in bone marrow exist. Bone marrow in normal post-natal life is confined to the interior of the bones in mammals. Since this tissue which is capable of elaborate synthesis in the formation of blood and antibacterial substances, has a sinusoidal circulation and since the apatite salts of bone in a dry state provide efficient heat insulation, it might be anticipated that an elevation of temperature in bone marrow above the surrounding coverings would obtain. This elevation was not found.

Normal bone marrow of adult mammals is of two distinct types, erythropoietic or red marrow and fatty or yellow marrow. E. Neumann (1) showed that in normal adult man red marrow was exclusively limited to bones of the body-trunk and proximal portions of the limbs while the distal extremity bones contained only yellow marrow. Moreover, he demonstrated a congruence of marrow arrangement in upper and lower extremities. This proximal-distal relationship of red-yellow marrow also has been observed in many other mature mammals and birds by other workers. The cause of this topographical phenomenon is unknown but led to particular study of thermal conditions in extremity marrows.

Direct observations were made of intramedullary temperatures in rabbit, rat and pigeon. Where the bones were thin and small, temperature of the marrow was indirectly estimated by comparing superficial and deep periosteal temperature at immediately opposing loci.

Further indirect readings in very young rats were obtained by determining daily the deep body temperature immediately following removal from the nursing mother and again after short exposure to room temperature. This was done since Pembrey (2) established the fact that newly born rats were incapable of maintaining a constant temperature, in order to find out how nest life and exposure affected the temperature at which

[1] This study was aided by a grant from the Douglas Smith Foundation for Medical Research and from the Committee on Scientific Research of the American Medical Association.

bone marrow was working and also to study quantitatively the development of homeothermism in this animal.

METHODS. The temperature determinations were made by means of thermo-electric couples of copper and constantan and a d'Arsonval galvanometer (Leeds and Northrup, type R) which had a sensitivity of 0.0049 micro-ampere per millimeter, at 1 meter distance, with appropriate resistance so that a deflection of 1.9 cm. per 1°C. was obtained. The fixed junctions were maintained in a Sheard (3) constant temperature bath. Readings were obtained by the use of two types of variable junctions. All of the intramedullary determinations were made with junctions of insulated no. 34 gauge copper and constantan wire twisted together for 8 cm. and soldered at the tip where insulation was removed; these wires were marked at 1 cm. intervals with paint to determine the length of wire inserted in the tissue. Indirect readings as used in the skull, tail, and in the peritoneal cavity were obtained by couples composed of no. 44 gauge copper and constantan insulated wire inserted and soldered in no. 28 gauge steel hypodermic tubing beveled at the end and provided with a bakelite handle.

Observations were made in adults on 20 rabbits, 10 albino rats, and 10 pigeons. The animal was comfortably fastened in an animal holder and observations immediately commenced. In young rabbits and rats it was the practice to insert a no. 18 gauge steel hypodermic needle through the subcutaneous tissues and then with a slight boring motion directly into the marrow cavity, and through this the twisted couple was inserted into the marrow. In old rabbits the bone was found too dense for this procedure and here using 1 per cent procaine hydrochloride as a local anesthetic a tiny incision was made, the bone was drilled and the junction inserted into the marrow through the drill hole. The superficial tissue was then rapidly united with a silk suture.

As soon as the junction was inserted a reading of the temperature was taken; this was considered important to offset possible circulatory damage by the mechanical procedure involved in introducing the wires. The junctions were inserted as far from the point of entrance as possible to minimize the disturbing effects of the local anesthetic and handling. Readings were made of the intramedullary temperature of humerus, radius, ulna, femur, tibia, metatarsus, and phalanges in the rabbit and of the femur and tibia of the other animals used. Indirect readings were taken on the outside and inside of the rabbit and rat skull and sternum at immediately opposing points, and in the periosteum of the bones of the tail in rabbit and rat, and upper extremity of the pigeon.

Always the more peripheral bones were investigated first and the plan involved insertion of the junction first in the marrow cavity of the phalanx with immediate reading of the temperature, and then similarly for other

bones in the following order: metatarsus, tibia, femur, radius, ulna, and humerus, as the thermocouples were inserted; all the thermocouples were left in place until the end of the experiment, and temperatures were determined every 5 minutes.

Several precautions were found necessary. Shaving was not done and handling was kept minimal. The experiment was started as soon as the animal was placed on the table; the animals were fastened in place by adhesive strips across the toe nails to avoid a tourniquet effect. Determinations were made in a small quiet room where the temperature was maintained between 24 and 26°C. At the end of each experiment the animal was killed while on the animal board and the extremities x-rayed to ascertain the exact position of the wires; only in this way could coiling of the thermocouples be detected.

TABLE 1

*Temperature readings made on bone marrow in the bones of the legs of 6 resting rabbits, as rapidly as thermocouples could be inserted*

Only one thermocouple was inserted in each bone; one determination was made on right femur and the other on left femur, etc.

| | 2-5 | 2-7 | 2-12a | 2-12b | 2-18 | 1-31a |
|---|---|---|---|---|---|---|
| Femur marrow near hip joint | 38.85 | 39.2 | 38.65 | 38.35 | 39.3 | 38.1 |
| Femur marrow near knee joint | | 39.2 | 37.9 | 37.75 | 37.8 | 36.3 |
| Tibia marrow near knee joint | 38.2 | 38.75 | 37.6 | 37.5 | 38.65 | 34.25 |
| Tibia marrow near ankle joint | 37.5 | 38.15 | 34.6 | 35.6 | | 34.1 |
| Marrow of metatarsal bones | 35.65 | 37.9 | | 36.7 | 36.45 | |
| | 35.8 | 37.75 | | | 36.5 | |
| | | 37.65 | | | 36.25 | |
| | | 37.55 | | | | |
| Marrow of phalanx bones | | | 33.9 | 35.9 | 36.1 | |
| | | | 33.2 | 36.0 | 35.0 | |

RESULTS. In table 1 are shown initial readings made in 6 quiet rabbits, as rapidly as the thermocouples could be inserted. In each of these experiments the thermocouples were in place and readings made in less than 18 minutes. The upper femur and humerus marrow temperature was found equal to or slightly lower than (less than 0.3°C.) that of the peritoneal cavity. Progressing from these points distally to the phalanges there was found a temperature decrement of 1.5 to 6°C. in the resting rabbit. The temperature of the marrow in the tibia was found in every case less than that in the region of the principal blood vessels at the same level. In figure 1 are shown readings obtained at the time the junctions were inserted and also values at the same loci after 1 hour on the animal board. In each case the upper femur marrow temperature rose or fell several tenths of one degree or remained constant; the temperature of the

outlying bones always fell, the distal areas more than the proximal and a differential of 6 to 8°C. was frequently observed between the proximal and distal loci. It must be emphasized, however, that the decrement was not strictly linear; occasionally violent muscular contractions would cause an elevation of muscle and marrow temperatures of 2 to 4° above the quiet level.

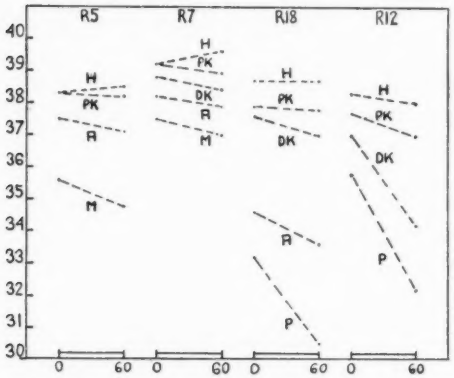


Fig. 1

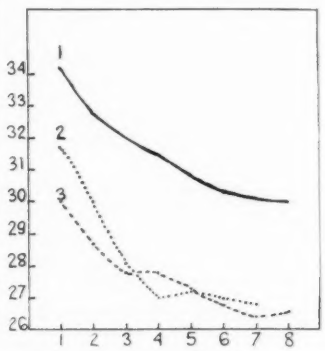


Fig. 2

Fig. 1. Thermal values obtained in the marrow of the lower extremities of the resting rabbit immediately on insertion of the thermocouple and one hour later, in degrees centigrade. The broken lines connecting these determinations merely indicate the trend of the temperature change. Legend: *H* = proximal femur, marrow; *PK* = distal femur; *DK* = proximal tibia; *A* = distal tibia; *M* = metatarsal; and *P* = phalanx. Ordinates, degrees C. Abscissae, time in minutes.

Fig. 2. The temperature gradient observed in the periosteum of the caudal vertebrae of 3 rats. Abscissae, distance from anus in centimeters; ordinates, temperature in degrees C. For each animal simultaneous data obtained are as follows. Intraperitoneal temperature; rat 1, 37.5°; rat 2, 39.1°; rat 3, 38.5°; mid-femur temperature, rat 1, 35.9°; rat 2, 38.25°; rat 3, 36.4°; mid-tibia temperature, rat 1, 34.9°; rat 2, 34.5°; rat 3, 34.3°.

In table 2 are given similar data obtained in 10 pigeons; the values are at a higher level than in the rabbit, and a slighter gradient may be seen between proximal and distal marrow temperature.

In figure 2 is shown the thermal decrement in the periosteum of the tail vertebrae of 3 mature albino rats. These data are presented because all of the distal bones of this appendage in these animals were filled with fatty marrow, while 1 or 2 proximal tail vertebrae were the site of erythropoiesis.

The temperature of cranial and sternum marrow, measured indirectly as stated, in many determinations in rat and rabbit was found to be identi-

cal with deep peritoneal temperature. These bones also were not subjected to the marked cooling that occurred in the extremities during rest and were maintained at warm internal body temperature.

In studying the development of homeothermism in the rat, daily observations were made on 15 rats of the peritoneal temperature immediately after removal from the next and again after 10 minutes at room temperature (24–26°). Daily puncture of the abdomen produced no apparent changes in the rats. In table 3 are shown values for 1 rat, typical of the

TABLE 2

*Values obtained in 10 mature pigeons, immediately after inserting thermocouples*

The data for upper extremity were obtained in the periosteum, for the lower extremity indirectly in bone marrow.

| | PIGEON I | PIGEON II | PIGEON III | PIGEON IV | PIGEON V* | PIGEON VI | PIGEON VII | PIGEON VIII | PIGEON IX | PIGEON X |
|---|---|---|---|---|---|---|---|---|---|---|
| Deep pectoral muscle | 42.2 | 41.6 | 42.3 | 41.1 | 43.95 | 41.0 | 41.4 | 41.7 | 41.4 | 39 65 |
| Shoulder, on humerus | 42.5 | 41.0 | 42.3 | 41.4 | | | | | 41.4 | |
| Elbow, on humerus | 41.0 | 40.3 | 41.7 | 40.05 | | | | | 40.5 | |
| Wrist, on radius | 40.4 | 38.0 | 40.3 | 40.05 | | | | | 39.2 | |
| Femur marrow at hip | 41.8 | 41.6 | 42.3 | 40.9 | 42.8 | 40.6 | 41.4 | 41.5 | 41.1 | 38.5 |
| Femur marrow at knee | 41.4 | 41.0 | 41.5 | 41.0 | 42.4 | 40.3 | 41.25 | 41.5 | 40.9 | 38.0 |
| Tibia marrow at ankle | 38.3 | 38.5 | 38.1 | 38.05 | 42.0 | 38.6 | 39.1 | 40.05 | 38.05 | 34.85 |
| Metatarsal periosteum | | | | 36.0 | 37.3 | 37.1 | 37.25 | 38.5 | 36.0 | 31.2 |
| Phalanx periosteum | | | | | 35.8 | 34.9 | 33.75 | 35.1 | | |

* Taken 5 minutes after exercise (flight).

TABLE 3

| | DAYS AFTER BIRTH | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ½ | 1 | 3 | 5 | 6 | 7 | 8 | 9 | 11 | 12 | 14 | 15 | 16 | 17 | 18 |
| Intraperitoneal temperature on removing from nest, degrees C | 36.0 | 36.0 | 35.5 | 35.7 | 36.3 | 34 4 | 36.0 | 36 5 | 35.2 | 36.5 | 35.3 | 36 0 | 36.0 | 36.4 | 36.9 |
| Intraperitoneal temperature 10 minutes later exposed to room temperature, degrees C | 27 5 | 27.5 | 27 8 | 28 8 | 30.1 | 30.1 | 31.0 | 32 8 | 33 6 | 34 5 | 34 2 | 34 6 | 35 0 | 36.4 | 36 9 |
| Room temperature, degrees C | 26.0 | 26 0 | 26 0 | 25 0 | 25.0 | 25 0 | 26 0 | 26 0 | 26 0 | 25 5 | 25.5 | 24.3 | 25 0 | 25 5 | 25 0 |

group. An inability to maintain a normal body temperature under these conditions was found to persist for 17 to 19 days after birth, although gradual improvement began on the 5th to 7th day after birth. Shaving the animal completely on the 19th day did not alter the thermal picture, showing that fur insulation was not the chief factor in maintaining homeothermy.

Discussion. The thermal gradient found in bone marrow of the extremities of rabbit, rat, and pigeon is evidence that the apatite salts of

bone in the wet state provide poor heat insulation. This is further supported by the fact that the heat of muscular activity of the limbs is reflected in an increase of marrow temperature.

The thermal gradient found in the limbs is of course not peculiar to bone marrow. Claude Bernard (4) found that the muscles of the limbs were cooler than the abdominal cavity. The data of Benedict, Miles, and Johnson (5) clearly show a peripheral gradient in the soft tissue of the extremities in man as do those of Loewy and Dorno (6), Bazett and McGlone (7), Morton and Scott (8), Eddy and Taylor (9), Coller and Maddock (10) among others. Moreover Bazett (11) stated that the temperature of the whole mass of the limbs at rest may be much below that of the rectum.

These data show that the bone marrow in the extremities, in contradistinction to that of the skull and sternum and other deep bones, is subject to the same cooling as occurs in other soft tissues in the extremities. It is felt that the chemical activities of the marrow do not importantly affect the thermal conditions in the face of an active circulation and poor bone insulation.

The temperature of the entire organism as well as the thermal condition of the bone marrow in an albino rat of an age less than 18 days is largely dependent on the environment, and is modified by nest temperature, huddling together of the young, and covering by the mother. The mechanism of what Deighton (12) has called the sub-poikilothermic state has been studied in a variety of animals by Pembrey (2), and Ginglinger and Kayser (13) from a metabolic standpoint.

## CONCLUSIONS

1. During muscular rest, a temperature gradient was found to exist in the bone marrow of the extremities of the rabbit, rat, and to a lesser extent in the pigeon. The more centrally located bones of the extremities together with cranial bones and sternum maintain a temperature similar to that of the peritoneal cavity. The cooling decrement is centrifugal in direction.

2. The temperature differential between central and outlying bone marrow at rest was usually 4 to 8°C. or more. No cooling was found in the bones of the skull and sternum under the same conditions.

3. The constant temperature mechanism in the albino rat was found established on the 17th to 18th day after birth.

The authors desire to express thanks to Dr. Paul C. Foster for aid in construction of the thermocouple apparatus.

## REFERENCES

(1) NEUMANN, E. Centralbl. f. d. med. Wissensch. **20:** 321, 1882.
(2) PEMBREY, M. S. J. Physiol. **18:** 363, 1895.
(3) SHEARD, C. Am. J. Clin. Path. **1:** 209, 1931.
(4) BERNARD, C. Leçons sur la chaleur animale, Paris. 1876.
(5) BENEDICT, F. G., W. R. MILES AND A. JOHNSON. Proc. Nat. Acad. Sci. **5:** 218, 1918.
(6) LOEWY, A. AND C. DORNO. Strahlentherapie **20:** 411, 1925.
(7) BAZETT, H. C. AND B. MCGLONE. This Journal **76:** 224, 1926.
(8) MORTON, J. J. AND W. J. M. SCOTT. J. Clin. Invest. **9:** 235, 1930.
(9) EDDY, H. C. AND H. P. TAYLOR. Am. Heart J. **6:** 683, 1931.
(10) COLLER, F. A. AND W. G. MADDOCK. Ann. Surg. **96:** 719, 1932.
(11) BAZETT, H. C. Physiol. Rev. **7:** 537, 1927.
(12) DEIGHTON, T. Physiol. Rev. **13:** 427, 1933.
(13) GINGLINGER, A. AND C. KAYSER. Ann. de Physiol. **5:** 710, 1929.

# LOCALIZATION OF THE CENTRAL RESPIRATORY MECHANISM AS STUDIED BY LOCAL COOLING OF THE SURFACE OF THE BRAIN STEM[1,2]

HAYDEN C. NICHOLSON

*From the Department of Physiology, University of Michigan, Ann Arbor*

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In 1883 Fredericq, working on rabbits, found that local cooling of the posterior portion of the floor of the fourth ventricle might depress or even arrest respiration. Respiration ceased, he found, with the chest in the expiratory position. Except for some experiments of Dittler (1912) upon the effects of central cooling upon the diaphragmatic action currents of rabbits, and the experiments of Gurdjian who in 1927 reported entirely negative results upon dogs, very little further work has been done upon the effects of cooling the brain stem on respiration. Considering Gurdjian's discordant results we felt that further study of the subject might be worth while, particularly since if local cooling was found to produce definite effects on respiration it might offer an additional means of investigating the localization and mode of action of the central respiratory mechanism. Such a method while of course having the disadvantage that its effects would probably be limited to more or less superficial structures would seem to have the advantages over the widely used section method that its degree and presumably the magnitude of its effects could be regulated and that there would probably be less interference with other vital functions.

In all our experiments we have used dogs anesthetized with morphine and urethane. The animal was placed on the table, back uppermost with the head rigidly held in a flexed position by a specially designed holder. By reflecting the posterior cervical muscles and those over the occiput, cutting through the atlanto-occipital ligament and the meninges, chipping away the occipital bone for a few millimeters on either side of the midline and slightly elevating the vermis of the cerebellum it was possible to expose a considerable portion of the fourth ventricle with practically no hemorrhage.

In the first few experiments a thin walled rubber tube of about 1 mm. bore was laid on the surface of the medulla with its tip one or two centi-

[1] Preliminary Report: This Journal, Proc. **101:** 80, 1932 and Proc. **109:** 81, 1934.

[2] This study was supported in part by a grant from the Rockefeller Foundation.

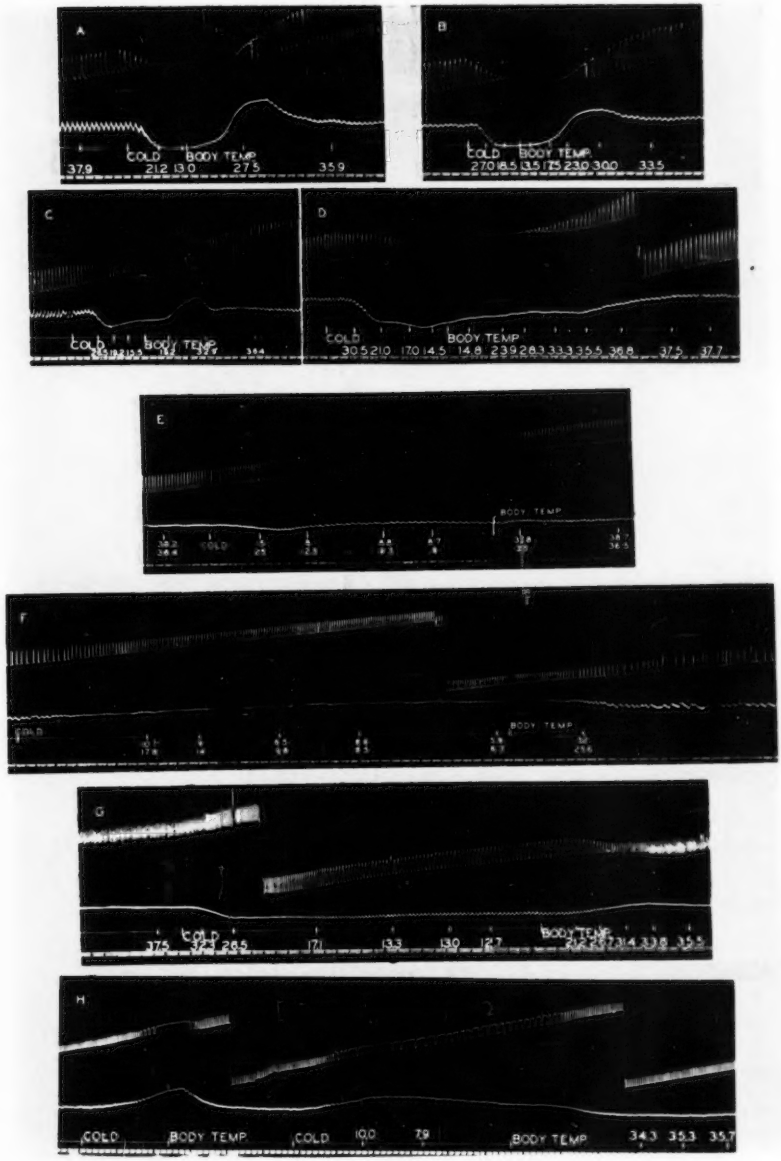
meters anterior to the apex of the calamus scriptorius. This tube was connected through a Y-tube with two flasks containing Locke's solution, the solution in one being kept at body temperature while that in the other was cold. The Locke's solution thus circulated over the medulla and accumulated near the posterior end of the incision from where it was removed by suction. A small thermometer was inserted through a T-tube into the ingoing stream and another was placed in the fluid just above the floor of the fourth ventricle. The area of the brain stem over which the solution circulated was of course rather extensive, though its exact extent could not readily be determined. Figures 1A, B, C, and D illustrate the effects of substituting the cold Locke's solution for that at body temperature. The lowest record is time in seconds, ten second and one minute intervals being indicated by double and triple breaks in the record. Above this is a record of a signal magnet used to record the moment of changing from one solution to the other, and also the temperature at intervals. The temperatures recorded are in degrees centigrade and represent the average of the readings of the two thermometers. Above this is a record of arterial blood pressure and above this a record of respiration taken from a spirometer connected with a rebreathing tank from which the animal breathed room air. Upstroke represents inspiration.

From an examination of the records we see that substitution of cold Locke's solution for that at body temperature immediately depresses respiration and very quickly stops it completely. When respiratory arrest occurred the chest seemed to be in or at least approaching the expiratory position. In C the last two or three respirations before cessation of breathing although gradually decreasing in amplitude show a marked lengthening of the inspiratory phase as though perhaps some influence normally interrupting inspiration were no longer present. Now when we return to the body temperature solution and respiration recommences, in three of the experiments illustrated, A, B and C, there is a considerable period during which there is a gradual increase in inspiratory tonus with as yet no evidence of expiratory activity. In B there appear a few small quick respirations superimposed upon this prolonged inspiration. Although D fails to show this marked increase in inspiratory tonus on recovery there is some suggestion of it in the first two or three respirations which show a greater duration of the inspiratory than of the expiratory phase. It appears then that although this extensive cooling of the medulla will completely abolish both inspiratory and expiratory activity the former may be somewhat more resistant to and recovers more quickly from the effects of the cooling than the latter.

In all but a few of the earlier experiments the cooling was localized to a small region of the brain stem through the use of an instrument consisting of a formica tube to one end of which was attached a thin walled copper

tube about 3 cm. in length and 3 mm. in diameter. A copper tube of about 1 mm. bore extended through the formica tube and through the copper tube almost to its tip, making it possible to circulate water of any desired temperature through the copper tip. The instrument was mounted so that the tip of the copper tube was in light contact with the surface of the brain stem at any desired point. A small thermometer was inserted through a T-tube into the stream of water as it entered the instrument and another thermometer was placed in the outcoming stream. Either these two temperatures or the average of the two are recorded at intervals on the kymograph record. Ordinarily not more than about 4 sq. mm. of nervous tissue was in contact with the applicator.

A large number of experiments were carried out with the tip of the applicator just anterior to the obex, that is, in the region of the classical respiratory center—the "neoud vital" of Flourens. The effects of cooling this region while varying considerably in degree, probably due largely to slight variations in the exact position of the applicator, qualitatively showed the greatest consistency. Typically such cooling resulted in an increase in the duration of the inspiratory phase of respiration relative to the expiratory phase. This effect might be so great as to cause an inspiratory arrest lasting for one or two minutes, apparently exactly similar to the "apneusis" described by Lumsden (1923). On the other hand this change in the relative duration of inspiration and expiration might evidence itself merely as a decrease in the duration of the pause usually observed after each expiration, with little or no actual increase in the duration of inspiration. In various experiments various effects intermediate between those two extremes might be observed. It is readily seen that the respiratory rate might be either increased or decreased depending upon the magnitude of the cooling effect. If the cooling produces, as mentioned above, merely a decrease in the duration of expiration without prolonging the inspiratory phase of respiration the respiratory rate will of course be increased. If the animal's normal respiration is characterized by a rather long expiratory pause this increase in respiratory rate may be considerable. It may also happen that a marked change is produced in the relative durations of inspiration and expiration with little or no change in respiratory rate. It is frequently observed that the expiratory pause is largely or completely abolished and an inspiratory pause of about equal duration established. Thus the original respiratory record appears inverted. Of course as the inspiratory phase of respiration becomes further prolonged respiratory slowing appears, which as mentioned above may be very marked. It frequently happens that as the temperature is gradually lowered the animal's respiration passes successively through several of these stages. There may be acceleration gradually increasing in degree, then, as prolongation of inspiration begins to appear, gradually decreasing and finally slowing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37.5
37.7
E
BODY TEMP
F
COLD
BODY TEMP
G
COLD
37.5
32.3 28.5
17.1
13.3
13.0
12.7
BODY TEMP
H
COLD
BODY TEMP
COLD
10.0
7.9
BODY TEMP
34.3
35.3
35.7

Fig. 1

of respiration below the original rate. Figures 1E, F and H are examples of local cooling of the posterior part of the fourth ventricle of varying degrees of effectiveness. Figure 1H shows two examples of marked slowing of respiration with well developed apneusis. In the first example an apneusis had lasted over one minute when the cooling was discontinued. Figure 1E shows a slight acceleration followed by a definite slowing with respiration tending to be apneustic in type. Figure 1F shows a well maintained acceleration amounting to about 135 per cent. It may be noted that in this record the animal's normal respiration was characterized by an unusually long expiratory pause, shortening of this pause accounting for the acceleration. These typical results of cooling this region may be obtained after section of both vagi, section of both superior laryngeal nerves and denervation of both carotid sinuses.

All the effects thus far described may be explained by the depression of some expiratory mechanism. Further evidence that the depressant effects of cooling the posterior portion of the floor of the fourth ventricle are exerted predominantly or at least most readily upon expiration is provided by figure 1G. During the course of this experiment the animal developed a peculiar and abnormal type of breathing in which an inspiration when it had reached only about half its normal height was interrupted by a rapid spasmodic expiration which was then succeeded by a respiration of the normal height. This cycle was then repeated. Such a course of events might perhaps be explained by an increase in irritability or an increase in the activity of an expiratory mechanism. In this experiment local cooling of the region just anterior to the apex of the calamus scriptorius abolished these spasmodic expirations and restored the respiration to its original normal type. As the temperature continued to fall typical slowing with inspiratory prolongation resulted. On return to body temperature the breathing reverted to the abnormal type. In another experiment raising the temperature two or three degrees above body temperature was sufficient to initiate this abnormal type of respiration characterized by increased expiratory activity in an animal previously breathing perfectly normally. Return to body temperature resulted in a return to normal respiration.

The question may now be asked whether it is possible to produce any inhibition of *inspiration* by local cooling of the posterior portion of the floor of the fourth ventricle. It appears that it is, though it is ordinarily much less marked and less readily demonstrated than is inhibition of expiration. In the apneustic type of breathing so frequently produced by cooling it is seen that inspiration does not rise to its maximum as rapidly as normally, this being indicated on the record by a definite "slanting off" of the inspiratory tracing. This slanting becomes particularly noticeable as the maximum height is approached. This seems to suggest a less rapid building up of the inspiratory impulses. Cooling also usually results in a

decrease in the amplitude of respiration due apparently largely to a more shallow inspiration. Although local cooling as accomplished in these experiments seldom caused the death of the animal it did so occasionally and when death did occur it was usually preceded by a series of apneuses gradually decreasing in height so that the final apnea occurred with the chest in a position of passive expiration or in an intermediate position. Of course if death was preceded by a prolonged period of well developed apneustic breathing it might be suggested that the final apnea resulted from asphyxiation of the centers rather than specific depression of an inspiratory mechanism by cooling. However this was not the case, apnea when it did occur usually appearing rather soon after the first application of cold, after only a brief period of apneustic breathing, and when there seemed to be no evidence that the animal was in fact suffering from lack of oxygen.

Although we made no particular effort to determine the least temperature change necessary to produce an appreciable change in respiration, and although as we have stated, there was considerable variation in different experiments in the magnitude of the effects produced by a given temperature change, it was observed in some experiments that appreciable changes in respiration might be produced by a surprisingly slight drop in temperature. In one experiment a definite and perfectly typical respiratory change was brought about by a lowering of the temperature of the water circulating through the applicator of 3°C.—from 37°C. to 34°C. Such a slight drop in temperature is certainly not sufficient to block or even appreciably affect conduction in a nerve fiber but it might conceivably alter the rate of discharge of a nerve cell.

Various workers, of whom one of the first was Markwald (1880), have pointed out that in the normal control of respiration other portions of the brain stem than the "noeud vital" were involved. In 1923 Lumsden presented evidence derived from experiments involving sectioning of the brain stem at various levels for the existence of four distinct respiratory centers. These centers are 1, a gasping center just above the apex of the calamus scriptorius which Lumsden believes is purely a relic of some primitive respiratory mechanism and plays no part in normal breathing; 2, an apneustic center at the level of the striae acousticae which when unregulated produces a continued inspiratory tonus; 3, a pneumotaxic center in the upper pons whose function is to interrupt periodically the activity of the apneustic center and bring about normal rhythmical breathing; and 4, an expiratory center located between the gasping and apneustic centers which when expiration is active aids the pneumotaxic center in interrupting apneusis and bringing about expiration, but which plays little part in normal quiet breathing in which Lumsden believes expiration is largely passive.

With the above work in mind we attempted to investigate the effects

on respiration produced by local cooling of the more anterior portions of the brain stem. In various experiments the point of the applicator has been placed at numerous points from the obex to the upper part of the pons. The results of local cooling of these regions may be very briefly stated. As the applicator was moved forward from the region of the "noeud vital" the effects of cooling became progressively less. With the applicator 8 to 10 mm. anterior to the calamus we were more apt to get acceleration of respiration than well developed apneusis, exactly similar to the effect produced by a *slight* lowering of the temperature with the applicator in the more posterior position. Local cooling of the region of the striae acousticae was tried repeatedly with no evidence of inspiratory depression. As was stated at the beginning of this paper the effects of such local cooling are probably limited to more or less superficial structures and we do not consider this failure to observe respiratory effects from local cooling of the more anterior portions of the brain stem as important evidence against the existence of respiratory "centers" in those regions. In fact the work of Keller (1931) and others makes it appear quite certain that respiration is influenced by impulses from various higher centers. However, the fact that a marked apneusis may be brought about by a moderate cooling of a small area in the region of the calamus scriptorius seems at least to remove the necessity for the existence of a "pneumotoxic" center in the upper pons with the specific function of interrupting inspiration. It appears that inspiration is normally rhythmically interrupted by the activity of some mechanism located just anterior to the apex of the calamus scriptorius.

We are at a loss to explain the totally negative findings of Gurdjian (1927) who states that "in more than one instance we applied ice to the medulla for a period of 3 to 10 minutes" (with no effect). We found that placing a small piece of ice in the calamus would produce an apneusis lasting until the ice had melted.

Although we have been interested primarily in respiratory changes effects of local cooling of the floor of the fourth ventricle on heart rate and blood pressure may be worthy of mention. In about 90 per cent of the observations there was a rise in pulse rate which was sometimes as marked as that produced by sectioning of both vagi. With the vagi previously sectioned cooling usually caused no change in pulse rate though there was sometimes a slight rise. In about 65 per cent of the observations there was a fall in blood pressure. These usual results, a rise in pulse rate with a fall in blood pressure, can probably be explained by depression of both cardio-inhibitory and vasomotor centers. However, in about 32 per cent of the observations the blood pressure showed a rise, sometimes very marked. In about 3 per cent of the cases there was no change in blood pressure. These variations in the effects of cooling on blood pressure

were not obviously associated with variations in respiration. If the original pulse rate was unusually low, cooling would usually cause a marked acceleration which was apt to be associated with a rise in blood pressure. However there were cases in which a marked rise in blood pressure was associated with only a slight or moderate rise in pulse rate. This rise in blood pressure might result from the acceleration of the pulse in the absence of depression of the vasomotor center or perhaps from inhibition of some depressor mechanism assuming that such a mechanism possessing tonic activity exists.

## SUMMARY AND CONCLUSIONS

Slight or moderate local cooling of the floor of the fourth ventricle just anterior to the apex of the calamus scriptorius depresses expiration leaving inspiration largely unaffected. Depending on the degree of this expiratory depression, respiratory rate may be increased, decreased or unchanged. A lowering of the temperature of 3°C., from 37°C. to 34°C. may be sufficient to definitely modify respiration.

More marked cooling of this region may depress inspiration also, even to the point of complete apnea and death.

It appears then that in the region just anterior to the apex of the calamus scriptorius are located mechanisms for the control of both the inspiratory and expiratory phases of respiration. The fact that the expiratory mechanism is more easily depressed by cooling may mean that it is more superficially located or that the inspiratory mechanism is inherently more resistant to such depression than is the expiratory.

The effects of local cooling of higher levels of the brain stem were qualitatively similar to the effects of cooling the calamus region, but the magnitude of the effects varied inversely with the distance from the calamus. This is interpreted as evidence neither for nor against the existence of other respiratory centers located at higher levels though if such centers exist they appear to play relatively minor rôles.

Local cooling of the floor of the fourth ventricle ordinarily produces an increase in pulse rate, which may be associated with either a rise or fall in blood pressure, usually the latter.

## REFERENCES

DITTLER. Zentralbl. f. Physiol. **26:** 1175, 1912.
FREDERICQ. Arch. f. Anat. u. Physiol., Supplement Band **51:** 1883.
GURDJIAN. This Journal **82:** 261, 1927.
KELLER. This Journal **96:** 59, 1931.
LUMSDEN. J. Physiol. **57:** 153, 1923.
J. Physiol. **57:** 354, 1923.
MARKWALD AND KRONECKER. Arch. f. Anat. u. Physiol. 440, 1880.

# REFLEX PATHWAYS CONCERNED IN INHIBITION OF HUNGER CONTRACTIONS BY INTESTINAL DISTENTION[1]

JOE LALICH, WALTER J. MEEK AND R. C. HERRIN

*From the Physiological Laboratory, University of Wisconsin, Madison*

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Although gastric reflexes from chemical stimulation of the intestine have given rise to an extensive literature much less has been reported on the gastric reflexes due to mechanical stimulation. Cannon and Murphy (2) found by handling the intestines that gastric evacuation was much delayed. They thought the long reflexes were mostly responsible especially those over the splanchnic pathways. Brunemeier and Carlson (1) observed that inhibition of gastric tonus and hunger contractions followed stimulation of the intestinal mucosa with a glass rod. The reflex was reduced when both vagi and splanchnics were cut, but it did not disappear, indicating that local reflexes were also concerned. An inhibition both of tonus and gastric contractions was described by Pearcy and Van Liere (6) after distention of various parts of the alimentary canal. No analysis of the nerve connections was made but they attributed them to the splanchnics. In 1931 Thomas and Mogan (5) noted that duodenal stimulation resulted in a primary excitation of the pyloric sphincter which was followed by relaxation of the sphincter and inhibition of the contractions in the pars pylorica. This they appropriately named the "enterogastric reflex." More recently Thomas, Crider and Mogan (4) have carefully analyzed these responses. They believe that there is a myenteric stimulation of the pyloric sphincter and a long reflex inhibition of the pars pylorica. It was concluded that the long pathway was through the vagus nerves.

We have previously shown (3) that distention of a jejunal Thiry fistula loop in an otherwise intact animal may inhibit gastric tonus and motility. The present report deals with the successive elimination of the possible extrinsic pathways concerned in this reflex.

METHODS. Eleven dogs with Thiry fistulae in the upper jejunum were trained to swallow a nasal catheter tipped with a balloon and to lie quietly without restraint during a period of record taking. The catheter was attached to a water manometer and the open limb of this to a recording tambour. Intestinal distention was secured in the Thiry loop by inserting

[1] Made possible in part by a grant from the Wisconsin Alumni Research Foundation.

a balloon under air pressures of 70 to 130 mm. Hg. Records were taken 18 hours after the previous feeding. In vagotomized animals the stomach was emptied by a small dose of apomorphine. When the gastric response of an animal to intestinal pressure was repeatedly determined the nervous mechanisms were removed successively under aseptic conditions. After each operation the animal was allowed to recover fully and another study made of its gastric response to intestinal pressure.

The vagotomies were performed by resecting the seventh rib under positive ether anesthesia and sectioning the vagi above the diaphragm. The splanchnics, both major and minor, as well as the lumbar sympathetic chains, were removed in successive operations through lumbar incisions.


**Fig. 1. Inhibition of gastric motility following distention of a Thiry loop after vagi were cut.**

RESULTS. The normal gastric response to intestinal pressure was carefully studied in 7 of the 11 animals. In all of these, pressures of 70 to 100 mm. Hg in the Thiry loop either eliminated or reduced the hunger contractions. Type II contractions were more easily influenced by pressure than those of type I. Tonus was generally reduced particularly with type II but not invariably. In case the pressures were high and the animal especially sensitive the tonus occasionally increased as it preceded vomiting. The responses usually occurred about eighteen seconds after the pressures were applied. Figure 1, although for brevity the record serves another purpose, may be used to illustrate a normal response which it resembles in every way.

As may be seen in column 7, table 1, the vagi were sectioned above the diaphragm in 7 animals. In all of these, tested by repeated distentions, gastric inhibition was induced. The response in most vagotomized animals

was however peculiar in that after the application of pressure there was often a temporary augmentation preceding the inhibition. Figure 1 is a record illustrating these points. From this series of experiments it was

TABLE 1

*Inhibition of stomach contractions by intestinal distention*

| NO. OF EXPERIMENT | NORMAL | R. SPL. AND CHAIN CUT | L. SPL. AND CHAIN CUT | R. AND L. SPL. AND CHAINS CUT | VAGI, R. AND L. SPL. AND CHAINS CUT | VAGI CUT | VAGI AND R. SPL. CUT | VAGI AND L. SPL. CUT | VAGI, R. AND L. SPL. CUT |
|---|---|---|---|---|---|---|---|---|---|
| 1 | + | + | | + | − | | | | |
| 2 | + | | | | | + | | | |
| 3 | + | | | | | + | + | | − |
| 4 | | | | | − | | | | |
| 5 | | | + | + | − | | | | |
| 6 | + | | + | + | − | | | | |
| 7 | + | | | | | + | | + | − |
| 8 | | | | | | + | − | | − |
| 9 | | | | | | + | | + | − |
| 10 | + | | | | | + | + | | |
| 11 | + | | | | | + | | +? | − |

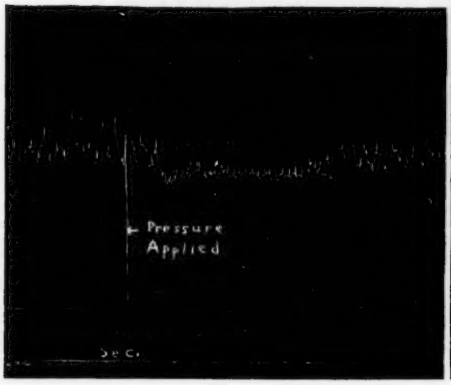


Fig. 2

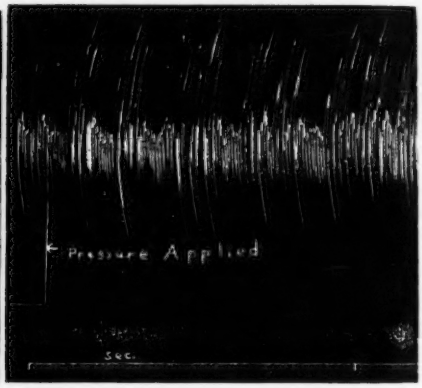


Fig. 3

Fig. 2. Inhibition of gastric motility following distention of a Thiry loop after both splanchnics and sympathetic chains were cut.

Fig. 3. Failure to secure inhibition of gastric motility on distention of a Thiry loop after section of vagi and splanchnics.

evident that other nerves, undoubtedly the splanchnics or lumbar chain, were functioning as reflex pathways.

Three animals were next studied following removal of the splanchnics on

both sides and section of the sympathetic chains at the level between the twelfth thoracic and first lumbar ganglia. The operations were carried out in two stages and tests were made following each operative procedure. In all three animals without exception reflex inhibition of the gastric contractions was still obtained. (See column 5, table 1.) Figure 2 illustrates one of these experiments. This series showed that nerves other than the splanchnics and lumbar chains, in all probability the vagi, could function as reflex pathways.

If either the vagi or splanchnics can serve as a reflex pathway, their elimination should result in abolition of the reflex unless the lumbar chains also function. In 5 animals therefore both vagi and both splanchnics were removed. (See column 10, table 1.) Although the stomachs still exhibited marked hunger contractions, distention of the intestine for prolonged periods with high pressures in no case produced inhibition. Figure 3 is a record from one of these animals. This series proved that both vagi and splanchnics may act as reflex pathways and that the sympathetic chains were not concerned. These results were confirmed by another series of 4 animals in which vagi, splanchnics and chains were removed. (See column 6.) Distention again produced no results.

Since the reflex may be mediated by either the vagi or splanchnics it is obvious that both sets of nerves contain afferent fibers from the intestine and efferent fibers to the stomach. That either the right or left splanchnic can function may be seen from the results presented in columns 8 and 9 of figure 1. Our experiments do not allow any distinction between right and left vagi.

Reflex inhibition of the stomach is more easily induced over the parasympathetic pathways than the sympathetic. While inhibition occurred in every case after vagotomy it did not take place as rapidly as it did after removal of the splanchnics. A comparison of figures 1 and 2 will illustrate this point.

The results here described differ somewhat from those of Thomas, Crider and Mogan (4). These authors however were dealing with relaxation in the pars pylorica and their reflex may well be mediated only by the vagus. Inhibition of the stomach from noxious bodily stimuli is thought to pass chiefly by way of the splanchnics. In our experiments gastric inhibition from distention of isolated intestinal loops may be mediated by either set of nerves.

## SUMMARY

Hunger contractions were recorded in dogs by the balloon method before and after distention of Thiry jejunal loops. Distention of a loop by pressures of 70 to 130 mm. Hg inhibits gastric tonus and motility. By successive surgical sections of the vagi, splanchnics and sympathetic chains it

was demonstrated that the reflex gastric inhibition could be mediated either by the vagi or splanchnics. Each set of nerves contained both afferent and efferent fibers. The evidence indicates that inhibition is more easily induced over the vagal pathways.

## REFERENCES

(1) BRUNEMEIER, E. H. AND A. J. CARLSON. This Journal **36:** 191, 1914.
(2) CANNON, W. B. AND F. T. MURPHY. J. A. M. A. **49:** 840, 1907.
(3) HERRIN, R. C., W. J. MEEK AND T. J. MATHEWS. This Journal **105:** 49, 1933.
(4) THOMAS, J. E., J. O. CRIDER AND C. J. MOGAN. This Journal **108:** 683, 1934.
(5) THOMAS, J. E. AND C. J. MOGAN. Proc. Soc. Exper. Biol. and Med. **28:** 968, 1931.
(6) PEARCY, J. F. AND E. J. VAN LIERE. This Journal **78:** 64, 1926.

# METABOLIC ASPECTS OF THYROID-ADRENAL INTERRELATIONSHIP[1]

S. B. BARKER, J. F. FAZIKAS[2] AND H. E. HIMWICH[2]

*From the Laboratory of Physiology, Yale University School of Medicine*

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Evidence has been accumulating in favor of an adrenal-thyroid interrelationship ever since the suggestion of Eppinger, Falta and Rudinger in 1908 (4) concerning the possibilities of dependent metabolic effects of thyroid, adrenal and pancreas. Cannon and Cattell (3) definitely linked thyroid secretion with adrenal activity. Very soon afterward, Levy showed that the thyroid secretion rendered more excitable the sympathetic structures acted on by adrenin in raising blood pressure (6). Moreover, the hyperthyroid heart has been found extremely sensitive to adrenin (8, 9).

As far as concerns metabolic aspects, Marine and his co-workers have been the principal proponents of adrenal-thyroid interrelationship (7). They observed that subcutaneous injections of adrenin produced the same percentage increases in the oxygen consumption and respiratory quotients of thyroidectomized rabbits as of normals. The actual rise was less in the former, but the lowered basal rate raised the percentage changes to normal. Boothby and Sandiford decided that the calorigenic action of adrenin was not consistently different in hyperthyroid, hypothyroid, or normal thyroid cases (2). These findings were opposed to the more extensive work reported by Tompkins, Sturgis and Wearn (10). Aub, reverting to adrenalectomy experiments, claimed that the presence of neither gland was necessary for the other to exert its normal effects (1).

EXPERIMENTAL METHODS. The present experiments performed on rats were undertaken to differentiate adrenin effects according to varying thyroid activity. The respiration apparatus used was the open system of Haldane especially adapted for work on small animals (5). As proof of the accuracy of the apparatus, twenty alcohol checks were run during the course of the work, yielding 0.672 as an average, with 0.013 as the maximum deviation from the theoretical 0.667.

Rats were kept for one week on a standard diet (Baloration), and their metabolism was determined in the post-absorptive condition. Adrenin

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[2] Now at Albany Medical College, Albany, N. Y.

was then injected subcutaneously, 0.5 cc. of the 1–1000 solution of Parke-Davis adrenalin preparation per kilo of body weight, and the animal immediately replaced in the chamber of the respiratory apparatus. The respiratory data were collected every two hours thereafter for several periods, and then for longer periods until the oxygen consumption returned to the basal level. These animals were returned to the diet for a few days and then, under deep nembutal anesthesia, the entire thyroid gland was extirpated through a simple tracheal approach as quickly as was compatible with minimal bleeding. No effort was made to leave parathyroids,

TABLE 1

*Effects of adrenin on respiratory metabolism of rats*

| | BASAL | HOURS AFTER ADRENIN | | | | |
|---|---|---|---|---|---|---|
| | | 2 | 4 | 6 | 10 | 14 |
| Normals—7 exper. on 7 animals: | | | | | | |
| $O_2$ mgm./hour | 417 | 476 | 517 | 500 | 449 | 424 |
| $CO_2$ mgm./hour | 412 | 574 | 508 | 508 | 427 | 412 |
| R.Q. | 0.72 | 0.88 | 0.71 | 0.67 | 0.69 | 0.71 |
| Thyroidectomized—10 exper. on 8 animals: | | | | | | |
| $O_2$ | 323 | 252 | 298 | 305 | 312 | 319 |
| $CO_2$ | 312 | 351 | 300 | 300 | 312 | 314 |
| R.Q. | 0.70 | 1.01 | 0.73 | 0.72 | 0.73 | 0.72 |
| (Unoperated, rendered hyperthyroid by feeding desiccated thyroid) 4 exper. on 4 animals: | | | | | | |
| $O_2$ | 625 | 1080 | 1054 | 844 | 708 | 658 |
| $CO_2$ | 635 | 1206 | 983 | 787 | 654 | 638 |
| R.Q. | 0.74 | 0.81 | 0.68 | 0.68 | 0.67 | 0.71 |
| (Thyroidectomized, rendered hyperthyroid by feeding desiccated thyroid) 8 exper. on 8 animals: | | | | | | |
| $O_2$ | 569 | 841 | 791 | 669 | 564 | 510 |
| $CO_2$ | 559 | 944 | 779 | 661 | 584 | 513 |
| R.Q. | 0.71 | 0.82 | 0.72 | 0.72 | 0.75 | 0.73 |

but the diet given the rats contained ample vitamin D, and calcium was added (as lactate). No tetany was observed in any of the animals.

After several days, the adrenin injection was repeated on these thyroidectomized rats. After a similar interval, these animals were then rendered hyperthyroid by the addition to the diet of a regulated amount of desiccated thyroid (Armour's). The effects of the same relative doses of adrenin were later obtained under this new hyperthyroid condition. As controls, a group of normal animals was subjected to the same procedure, omitting the thyroidectomy.

Table 1 contains the average results of the various types of experiments.

DISCUSSION. From the results it is evident that the metabolic response of the rat to adrenin injection is affected by changes in the thyroid activity. Hyperthyroidism induced by feeding desiccated thyroid, whether to a normal animal or to a thyroidectomized one, causes a tremendous increase in the adrenin stimulation ordinarily encountered. The oxygen consumption of the normal animals in only one case increased as much as 40 per cent over the basal, while after thyroid feeding a 60 to 70 per cent increase was the usual occurrence, with two instances of increases to 90 per cent or higher. In marked contrast to these rises in metabolism, the thyroidectomized rats always exhibited a drop, sometimes as great as 60 per cent of the basal rate.

The significance of the changes in oxygen consumption is not easily evaluated even in the normal animal, due to controversial evidence. It is, however, widely agreed that adrenin does have a calorigenic action, and this work adds support to the conception that the effect depends directly upon the degree of thyroid activity. The weight of the evidence indicates that the thyroid does not depend upon the adrenals for its metabolic changes. On the contrary, it now appears as though the adrenal exerts its calorigenic effects principally through the thyroid.

So important is this coördinated action that in the hypothyroid animal, adrenin alone is not powerful enough to exhibit the effects it does on the normal animal. In fact, the severely depressed oxygen consumption after thyroidectomy suggests the possibility that adrenin has even become toxic. However, if the thyroidectomized rat be given desiccated thyroid, the adrenin effect returns with all its former intensity, or even with the increased intensity of the unoperated hyperthyroid.

It has been suggested that part of the increased metabolism following the injection of adrenin may be caused by the greater muscular activity. If this is true, then the decrease in metabolic rate observed in the hypothyroid rats after injection of adrenin occurs despite the increased muscular exercise.

In the normal and hyperthyroid rats the quotients rose despite the greater $O_2$ consumption because the output of $CO_2$ increased even more. The significance of the respiratory quotients is doubtful, since part of the $CO_2$ eliminated may have been due to the displacement of preformed $CO_2$ by lactic acid as indicated by the succeeding compensatory fall of the quotient. On the other hand, in the thyroidectomized rats despite a production of $CO_2$ smaller than that of the other two groups of animals, the respiratory quotients rose to extraordinary levels due to decreased $O_2$ consumption.

## SUMMARY

1. The effects of adrenin on the respiratory metabolism of normal, hyperthyroid, and thyroidectomized rats were studied. The rise in oxygen

consumption shown by normal animals following adrenin injection was augmented after thyroid feeding, while in thyroidectomized animals a decrease of $O_2$ uptake was observed. Desiccated thyroid fed to thyroidectomized rats caused a return to the normal adrenin response.

2. These results indicate that the medulla of the adrenal gland exerts its calorigenic effect largely through the thyroid.

## REFERENCES

(1) Aub, J. C., E. M. Bright, J. Forman and J. Uridil. This Journal **61:** 300, 326, 349, 1922.
(2) Boothby, W. M. and I. Sandiford. This Journal **66:** 93, 1923.
(3) Cannon, W. B. and McK. Cattell. This Journal **41:** 39, 58, 74, 1916.
(4) Eppinger, H., W. Falta and N. Rudinger. Ztschr. f. klin. Med. **66:** 1, 1908.
(5) Haldane, J. J. Physiol. **13:** 419, 1892.
(6) Levy, R. L. This Journal **41:** 492, 1916.
(7) Marine, D. and E. J. Baumann. J. Metab. Res. **2:** 1, 1922.
(8) McDonald, C. H., W. L. Shepeard, M. F. Green and A. F. De Groat. This Journal **112:** 227, 1935.
(9) Rosenblum, H., R. G. Hahn and S. A. Levine. Arch. Ind. Med. **51:** 279, 1933.
(10) Tompkins, E. H., C. C. Sturgis and J. T. Wearn. Arch. Int. Med. **26:** 269, 1919.

# THE SPECIFIC DYNAMIC ACTION OF PROTEIN IN PANCREATIC DIABETES

G. C. RING

*From the Department of Physiology in the Harvard Medical School*

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The ingestion of fat by diabetic patients results in greater stimulation of metabolism than that occurring in normal subjects (see Bowen, Griffith and Sly, 1934). The same is true for partially depancreatized cats, provided the basal metabolism is about normal; but if the metabolism is greatly elevated as in severe diabetes, the stimulation due to fat disappears (Ring, 1935). Furthermore, the calorigenic action of carbohydrate is also absent in severe diabetes. It was of interest, therefore, to inquire whether the specific dynamic action of protein is absent or diminished when the metabolism becomes elevated as a result of pancreatectomy.

METHOD. The present study was made on almost completely depancreatized cats—animals in which all of the pancreas was removed except a small portion (about 10 per cent) lying close to the entrance of the main duct into the duodenum. These animals were given no insulin, yet they maintained their weight or lost it only gradually after several months. They all showed a glycosuria after a large meal.

The metabolism was measured as previously described (see Ring, 1935). The animal's basal rate was determined in the morning. About noon haddock was fed for the calorigenic studies. Frequently the animals ate 300 grams, but smaller quantities produced as satisfactory results. After eating and walking about for one hour, the cats were returned to the metabolism chamber and measurements of the changes in metabolism due to the protein ingestion were made during the second, third and fourth hours after the meal.

RESULTS. Normal cats, in spite of great variations in the intake of food at the previous meal, usually show differences of less than 10 per cent in metabolic measurements made 18 hours after eating. On the other hand, partially depancreatized cats may have basal metabolisms which vary as much as 40 per cent, depending upon the severity of the diabetes, the amount eaten at the previous meal and the duration of the fast prior to the metabolism measurements. Animals with a mild diabetes usually show normal metabolisms which can be elevated by withholding food for 48 hours and then feeding liberally, 18 hours before the next metabolism

measurement. If the diabetes is severe, the basal metabolism will be high in the postabsorptive state, but often this may be reduced by fasting for 48 hours. Occasionally a longer time is required. These are examples of the serious effects of large meals after fasting in diabetes (see Joslin, 1928) and the benefit derived from fasting when diabetes becomes severe (see Allen, 1914).

The procedures above described have been used to obtain at will either a high or low basal metabolism on which to superimpose the stimulation due to ingested protein. Cats 5 and 7 of the diabetic group usually had a normal basal metabolic rate which could be elevated by a period of fasting and a large intake of food during the afternoon prior to the metabolism measurements. Cat 8 once fell into this group, but at the time of the studies recorded in table 1, he, like 6 and 9, had a high basal rate unless he had fasted for at least two days.

In most cases the metabolism after the ingestion of haddock reached its maximum by the second hour. Occasionally three hours were required, but usually the measurements for the second, third and fourth hours were similar so that for uniformity all the figures are averaged.

Wide differences are to be seen in the calorigenic action of protein in different animals. Cats 6 and 8 of the diabetic group show very marked stimulation. This is due to undernutrition rather than diabetes, since cats 5 and 7, which had not lost weight, showed increases similar to those found in the normal cats. Furthermore, cat 6, when it had a body weight of 2.30 kgm., showed a calorigenic effect of 20.8 cal., but this stimulation amounted to 26.3 cal. when the body weight dropped to 1.95 kgm. All the other specific dynamic responses except one in this cat were inversely proportional to the weight changes. This is in accord with the observations of Gibbons (1924), who showed similar differences when a fat and a thin dog were compared. Again, Wilhelmj and Mann (1930) observed this after the administration of glycine and alanine to dogs which had fasted or been well fed.

The amount of haddock given varied between 150 and 300 grams. Yet the smallest intake produced as great a caloric response as larger amounts given to the same animal. Cats 1, 5, 6 and 8 illustrate this point. This means that in every case there was the maximal absorption and the greatest stimulation of metabolism which protein from haddock can produce.

All of the diabetic animals studied showed a marked increase in metabolism after protein ingestion, whether the basal rate was high or low. The increases were about the same in the two situations, except for cat 6. The differences in that animal, I believe, can be accounted for by changes in body weight, as explained above. On two occasions when the body weight was 2.1 kgm., the metabolic stimulation for the low metabolism was 19.3, and for the high metabolism was 22.4 cal.

The respiratory quotients during control periods are usually lower for the diabetic than the normal animals. The high basal metabolism in the

TABLE 1

*The metabolism of normal and diabetic cats before and after ingestion of protein*

| | CONTROL PERIOD | | HADDOCK GIVEN GRAMS | 2ND HOUR AFTER | | 3RD HOUR AFTER | | 4TH HOUR AFTER | | AVERAGE INCREASE | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| NORMAL CATS | R.Q. | Calories per square meter per hour | | R.Q. | Cal. | R.Q. | Cal. | R.Q. | Cal. | Cal. | Per cent |
| 1 | 0.77 | 28.1* | 150 | 0.77 | 38.2 | | | 0.83 | 42.5 | 12.2 | 43 |
| | 0.78 | 28.3 | | | | | | | | | |
| 1 | 0.79 | 28.5† | 300 | | | 0.77 | 42.9 | | | 14.2 | 49 |
| | 0.80 | 28.9 | | | | | | | | | |
| 2 | 0.79 | 26.0† | 180 | | | 0.80 | 37.1 | 0.78 | 38.4 | 11.1 | 42 |
| | 0.78 | 27.3 | | | | | | | | | |
| 3 | 0.80 | 29.1* | 300 | 0.75 | 43.3 | 0.76 | 44.8 | 0.82 | 43.6 | 14.8 | 51 |
| 4 | 0.74 | 31.2* | 230 | 0.79 | 44.3 | 0.80 | 46.5 | | | 15.3 | 49 |
| DIABETIC CATS | | | | | | | | | | | |
| 5 | 0.73 | 28.4* | 190 | 0.75 | 38.4 | 0.77 | 38.8 | 0.72 | 39.7 | 10.6 | 37 |
| 5 | 0.78 | 30.7* | 200 | 0.72 | 42.1 | | | 0.76 | 42.4 | 11.5 | 37 |
| 5 | 0.75 | 34.8‡ | 150 | 0.75 | 45.3 | | | 0.72 | 46.3 | 11.0 | 32 |
| | 0.75 | 34.8 | | | | | | | | | |
| 6 | 0.78 | 31.4† | 290 | 0.74 | 50.8 | | | | | 19.3 | 61 |
| | 0.78 | 31.5 | | | | | | | | | |
| 6 | 0.74 | 34.6† | 300 | 0.74 | 60.5 | | | 0.74 | 61.1 | 26.3 | 76 |
| | 0.71 | 34.3 | | | | | | | | | |
| 6 | 0.77 | 35.5* | 300 | 0.73 | 59.7 | 0.74 | 59.5 | 0.72 | 64.9 | 25.8 | 73 |
| | 0.75 | 35.8 | | | | | | | | | |
| 6 | 0.78 | 40.5* | 300 | 0.72 | 61.0 | 0.76 | 63.3 | 0.78 | 59.7 | 20.8 | 51 |
| 6 | 0.79 | 40.6* | 200 | 0.75 | 63.8 | 0.69 | 64.4 | 0.72 | 60.2 | 22.4 | 56 |
| | 0.79 | 40.2 | | | | | | | | | |
| 7 | 0.74 | 33.5* | 200 | 0.70 | 45.5 | 0.75 | 45.5 | | | 12.2 | 37 |
| | 0.77 | 33.2 | | | | | | | | | |
| 7 | 0.74 | 37.7‡ | 200 | 0.70 | 51.0 | 0.71 | 53.9 | 0.70 | ? | 14.8 | 39 |
| 8 | 0.70 | 32.8† | 290 | 0.67 | 46.4 | 0.70 | 51.6 | 0.71 | 51.7 | 17.8 | 56 |
| | 0.70 | 31.3 | | | | | | | | | |
| 8 | 0.77 | 38.3* | 150 | 0.76 | 56.8 | 0.77 | 60.4 | 0.76 | 57.1 | 19.0 | 49 |
| | 0.76 | 39.8 | | | | | | | | | |

* Fasted for 18 hours.

† Fasted for 48 hours.

‡ Fasted for 48 hours, then fed and fasted for 18 hours.

partially depancreatized cats is frequently accompanied by high quotients. This may be interpreted as dependent upon carbohydrate stores. Smaller

food intake or longer fasting means lower metabolism, less carbohydrate storage and thus lower respiratory quotients, and vice versa. After protein ingestion, the respiratory quotients continue lower in the diabetic animals than in the controls, suggesting that less of the potential sugar in the protein is being burned (see Ralli, Canzanelli and Rapport, 1931).

Discussion. After partial pancreatectomy in dogs, Pratt (1916) showed that there was very little disturbance in the absorption of nitrogenous material. The fact that the specific dynamic effect of protein in cats after a similar operation is not smaller than in normal animals is added evidence on this point.

It should be emphasized that the increase in metabolism after protein ingestion is the same whether the basal rate is high or low. If, as has been frequently suggested, the increased metabolism of diabetes were due to the calorigenic action of protein, with a high basal metabolism one would expect a diminution or disappearance of the specific dynamic effect. In other words, after protein ingestion which gives maximal stimulation of metabolism, the total metabolism should reach the same level whether the start was a high basal level or a low one. The only weakness in this argument is that possibly endogenous protein catabolism works through different channels than exogenous. However, Chambers and Lusk (1930) have some important evidence in this connection. In their dog 61, the normal basal metabolism was 21.8 cal. per hour; after phlorhizin, on one occasion the basal metabolism rose to 28.4 and on another to 24.8. The nitrogen excretion paralleled the metabolism changes. Giving of 300 grams of meat to this dog, while normal, stimulated the metabolism by 11.4 cal., but after phlorhizin only 4.3 and 5.1 cal. This dog had the same total metabolism after protein ingestion whether his basal metabolism was high or low. The above observation shows strikingly a difference between phlorhizin and pancreatic diabetes. The experiments on pancreatic diabetes support the earlier evidence of Ring and Hampel (1933). They showed that postaborptive excretion of nitrogen in depancreatized cats increased, but that this frequently occurred while the metabolism was normal and that it did not further increase when the metabolism became elevated.

## CONCLUSIONS

1. The previous intake of food is important in determining the basal metabolism of partially depancreatized cats.

2. The calorigenic action of protein in undernourished diabetic cats is greater than normal.

3. The calorigenic effect of protein is similar in diabetes whether the metabolism is high or low. This is in contrast to the disappearance of metabolic stimulation by fats and carbohydrates when the metabolism becomes elevated.

4. After protein ingestion the respiratory quotients are in general lower in the diabetic than in the normal animal.

5. These observations offer further support for other experiments showing that specific dynamic action of protein is not responsible for the increased metabolism of diabetes.

## REFERENCES

ALLEN, F. M. J. A. M. A. **63:** 939, 1914.
BOWEN, B. D., F. R. GRIFFITH, JR. AND G. E. SLY. J. Nutrition **8:** 421, 1934.
CHAMBERS, W. H. AND G. LUSK. J. Biol. Chem. **85:** 611, 1930.
GIBBONS, R. This Journal **70:** 26, 1924.
JOSLIN, E. P. Treatment of diabetes mellitus. Lea and Febiger, p. 159, 1928.
PRATT, J. H. This Journal **40:** 145, 1916.
RALLI, E. P., A. CANZANELLI AND D. RAPPORT. Ibid. **96:** 331, 1931.
RING, G. C. Ibid. **112:** 124, 1935.
RING, G. C. AND C. W. HAMPEL. Ibid. **105:** 300, 1933.
WILHELMJ, C. M. AND F. C. MANN. Ibid. **93:** 69, 258, 1930.

# THE SOURCE OF THE BLOOD ACETONE RESULTING FROM THE ADMINISTRATION OF THE KETOGENIC PRINCIPLE OF THE ANTERIOR HYPOPHYSIS

I. ARTHUR MIRSKY

*From The Department of Metabolism and Endocrinology, Institute for Medical Research, Jewish Hospital, Cincinnati*[1]

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The production of acetone bodies generally has been ascribed to a disturbance in the relative proportions of fat and carbohydrate that are being coincidentally oxidized by the tissues. Thus, it has been assumed that whenever there is a decrease in available carbohydrate (ketogenic diet or starvation), or in such conditions as phlorhizin and pancreatic diabetes, the oxidation of carbohydrate is decreased, and the oxidation of fat is correspondingly accelerated, the acetone bodies arising in consequence of an incomplete oxidation of the latter.

There is considerable evidence in the literature which indicates that the muscles can account for very little, if any, of the acetone bodies which accumulate in the blood. This may be exemplified by the studies of Chaikoff and Soskin (1) who showed that injected acetoacetic acid remained longer in the blood of depancreatized dogs than in normal dogs, despite the fact that the muscles of both types of animals utilized the acetone bodies with equal facility. They concluded that the extrahepatic tissues are not responsible for the production of ketones. Himwich, Goldfarb and Weller (2) analyzed the afferent and efferent blood of various organs, and found the liver to be the most constant source of acetone substances in phlorhizin and pancreatic diabetes. It has also been shown that in all conditions where ketosis is known to occur, the muscles continue to use carbohydrate. Thus, for example, Soskin and Mirsky (3) demonstrated that the muscles of the animal with "hunger diabetes" utilize glucose at a normal rate.

In view of the contradictions noted above, it seemed of interest to investigate a ketosis produced by means other than the production of diabetes or carbohydrate starvation. Within recent years, a number of investigators have demonstrated the presence of a ketogenic principle in extracts of the anterior lobe of the hypophysis. The ketosis induced by the administration of this principle has been ascribed to an increased oxidation of fat by all tissues (4). We utilized this means to produce ketosis and studied

[1] Aided by the David May Memorial Fund.

the effects of potent extracts of the anterior lobe of the hypophysis on the blood acetone in normal and in eviscerated rabbits. If the ketogenic principle stimulates a disproportionate use of fat at the expense of carbohydrate in the muscles, then significant effects should be obtained in the eviscerated animals as well as in the normals.

METHODS. Our study was carried out on white, male, New Zealand rabbits weighing approximately 2 kgm. All the animals were fasted for twenty-four hours. Nembutal[2] anesthesia was employed throughout all experiments to permit the withdrawal of blood samples from the exposed femoral artery. Evisceration was performed by a method somewhat modified from that of Drury (5). In some instances the kidneys were removed with the rest of the abdominal viscera, but since this procedure did not influence the results, it was subsequently eliminated.

The "total acetone" of the blood was determined by a modification of the Van Slyke and Fitz method (6), which enabled us to use 5 cc., instead of 10 cc., of blood for each determination. The error of a single determination is less than 1 mgm. per cent and, therefore, any value below that amount was regarded as zero.

An active neutralized alkaline extract made from powdered beef anterior pituitary lobes was used.[2] The minimal effective dose for the production of a definite rise in blood acetone was established to be the equivalent of two grams of fresh gland per kilogram of body weight. In some instances larger amounts of extract were administered to reveal the quantitative nature of the response in normal animals. Following the subcutaneous administration of the anterior hypophyseal extract, blood samples were drawn at hourly intervals for three hours in the normal rabbits. Since glucose was not administered to the eviscerated animals, blood samples were drawn usually for only two hours because these animals died in from $2\frac{1}{2}$ to 3 hours unless glucose was administered after the two hour interval. Six rabbits were used in each group.

It should be noted that the eviscerated preparations received relatively larger doses of extract than the normal controls because the dosage was estimated on the initial weight of the animals before evisceration. Hence, the final dose administered to these animals varied from $1\frac{1}{3}$ to twice that given to the normal rabbits.

RESULTS. The results are detailed in table 1. It may be noted that the normal animals respond to the subcutaneous injection of the ketogenic principle of the anterior hypophysis with a prompt appearance of acetone in the blood. This, in most instances, reaches a maximum in two hours and has begun to recede by the third hour. In no case is there a rise in the

[2] We are indebted to Dr. Edw. D. Campbell of the Eli Lilly Research Laboratories for generous supplies of Beef Anterior Pituitary Extract, and to the Abbott Laboratories for a supply of Nembutal.

acetone content of the blood of the eviscerated preparations in spite of the fact that they received relatively larger amounts of ketogenic principle. In several experiments, the liver was left intact while the other viscera were removed. Administration of the ketogenic principle to such animals results in a response similar to that observed in the unoperated normal rabbits.

Discussion. The injection of a neutralized alkaline extract of the anterior lobe of the hypophysis results in a rapid appearance and rise in the blood ketones of normal rabbits. Since this does not occur in the completely eviscerated animal, but does in the animal in which only the liver

TABLE 1

| RABBIT NUMBER | PERIOD OF FASTING | EQUIV. OF FRESH GLAND PER KILO | TOTAL BLOOD ACETONE | | | |
|---|---|---|---|---|---|---|
| | | | Control | After injection | | |
| | | | | 60 min. | 120 min. | 180 min. |
| | *hours* | *grams* | *mgm. per cent* | *mgm. per cent* | *mgm. per cent* | *mgm. per cent* |
| Control 1 | 24 | 2.0 | 0 | 8.5 | 17.0 | 20.5 |
| Control 2 | 24 | 2.0 | 0 | 21.0 | 33.0 | 9.0 |
| Control 3 | 24 | 2.0 | 0 | 25.0 | 13.0 | |
| Control 4 | 24 | 2.0 | 0 | 8.0 | 14.0 | |
| Control 5 | 24 | 2.0 | 0 | 10.0 | 14.5 | 12.5 |
| Control 6 | 24 | 2.0 | 0 | 12.5 | 18.0 | 13.5 |
| Eviscerated 1 | 24 | 3.0* | 0 | 0 | 0 | |
| Eviscerated 2 | 24 | 2.7* | 0 | 0 | 0 | |
| Eviscerated 3 | 24 | 3.3* | 0 | 0 | 0 | |
| Eviscerated 4 | 24 | 3.6* | 0 | 0 | 0 | |
| Eviscerated 5 | 24 | 4.0* | 0 | 0 | 0 | |
| Eviscerated 6 | 24 | 2.8* | 0 | 0 | 0 | 0 |

* Dose recalculated from the final weight of the preparation after removal of viscera.

remains intact, it is obvious that the ketogenic principle acts solely on the liver, and that the muscles do not contribute in any measurable degree to the accumulation of acetone bodies in the blood. That this ketosis is not due to a flushing out of ketogenic material from the liver is indicated by the observations of Butts, Cutler and Deuel (7). It is evident also that the accumulation of acetone in the blood of the normal rabbit is not dependent upon a lack of carbohydrate utilization by the muscles, since it was found that the eviscerated animal continues to remove glucose from its blood until hypoglycemia and death ensue.

It is now generally recognized that only glycogenic substances are anti-

ketogenic (8), and that a low liver glycogen is found in all conditions which are associated with ketonemia. When this is considered together with Deuel's observation that the ketogenic principle produces a marked depletion of liver glycogen (9), it becomes probable in the light of our observations, that this diminution of liver glycogen, (whether due to glycogenolysis or to carbohydrate starvation), is the essential factor in the formation of ketones by the liver. If we assume that the essential disturbance in all the above conditions is an acceleration of the rate of hepatic glycogenolysis, then it becomes possible to reconcile the apparent contradictions discussed in the introduction. A persistently increased glycogenolysis, (that is, an inability to retain glycogen) will result in a diminution in the glycogen concentration of the liver cells, and a consequent reduction in the carbohydrate available for oxidation by the liver itself. The gluconeogenesis from non-carbohydrate sources that is known to occur in these conditions (3) (10) may be a compensatory mechanism which, however, does not increase the carbohydrate tension of the cell because the rate of glycogenolysis remains excessive. Because of the decrease in carbohydrate available for oxidation by the liver cell itself, an increased catabolism of fat must occur irrespective of whether it is converted to glucose or directly oxidized. This increased breakdown of fatty acids in the presence of a *relative* diminution of carbohydrate utilization will lead to an incomplete catabolism of the end products of $\beta$ oxidation, and acetone bodies are formed (11).

This hypothesis is in accord with the concept that the oxidation of glucose is essential to the complete catabolism of the fatty acids, but relegates these processes to the liver, and obviates the erroneous assumption that there is a diminution in the utilization of carbohydrate by the extrahepatic tissues in all conditions in which acetone bodies are produced.

## SUMMARY AND CONCLUSIONS

1. The ketogenic principle of the anterior lobe of the hypophysis acts solely on the liver, and does not stimulate the oxidation of fat in the extrahepatic tissues.

2. The muscles do not contribute in any measurable degree to the accumulations of ketones in the blood.

3. An hypothesis is presented attributing the production of acetone bodies to a relative decrease of carbohydrate utilization by the cells of the liver in the presence of an increased catabolism of fatty acids consequent to an accelerated hepatic glycogenolysis.

We are indebted to Miss Dorothea Hamm for technical assistance.

## REFERENCES

(1) CHAIKOFF, I. L. AND S. SOSKIN. This Journal **87:** 58, 1928–29.
(2) HIMWICH, H. E., W. GOLDFARB AND A. WELLER. J. Biol. Chem. **93:** 337, 1931.
(3) SOSKIN, S. AND I. A. MIRSKY. This Journal **114:** 106, 1935.
(4) ANSELMINO, K. J. AND F. HOFFMAN. Klin. Wchnschr. **10:** 2380, 1931.
(5) DRURY, D. R. This Journal **111:** 289, 1935.
(6) VAN SLYKE, D. D. AND R. FITZ. J. Biol. Chem. **32:** 495, 1917.
(7) BUTTS, J. S., C. H. CUTLER AND H. J. DEUEL, JR. J. Biol. Chem. **105:** 29, 1934.
(8) SHAPIRO, I. J. Biol. Chem. **108:** 373, 1935.
(9) DEUEL, H. J. J. Biol. Chem. **105:** 19p, 1934.
(10) SOSKIN, S., I. A. MIRSKY, L. M. ZIMMERMAN AND N. CROHN. This Journal **114:** 110, 1935.
(11) SHAFFER, P. A. J. Biol. Chem. **47:** 449, 1921.

# THE INHIBITORY INFLUENCE OF THE ACIDITY OF THE GASTRIC CONTENTS ON THE SECRETION OF ACID BY THE STOMACH

CHARLES M. WILHELMJ, F. T. O'BRIEN AND FREDERICK C. HILL

*From the Departments of Physiology and Experimental Surgery, Creighton University School of Medicine, Omaha, Nebraska*

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Pavlov (1) suggested that the acidity of the gastric contents may inhibit further secretion of gastric juice. More recently several investigators (2–8) have suggested that acid in the stomach either inhibits further secretion of acid or changes the secretion to a non acid diluting fluid. It has also been stated (9) that removal of the continuous secretion every fifteen minutes augments the rate of secretion. The application of gastric juice and 2 per cent butyric acid have been found to markedly inhibit the continuous secretion of fundic pouches (10), while lactic acid stimulated. Vandorfy (11) found that both citric and acetic acids inhibited acid secretion and proposed that the principle of "acid inhibition" be used as a clinical test of the "secretory energy" of the stomach. Paul (12) observed that when 0.5 per cent hydrochloric acid solutions were placed in the stomachs of patients with gastric and duodenal ulcer, the continuous secretion was definitely inhibited, the degree of inhibition being directly proportional to the rate of the previous secretion.

Another group of investigators (13–18) have emphasized that the acidity of the secretion from fundic pouches and isolated stomachs remains constant in spite of the presence of acid contents and have therefore denied that acid inhibits or causes a non acid diluting fluid to be secreted. Likewise, we (19–24) have found that acid solutions (0.024 to 0.173 normal hydrochloric acid) when placed in fundic pouches or the intact stomach do not inhibit secretion when *histamine* is used as the stimulant. We (19) have demonstrated that unless some method is used to determine dilution of the acid solution it may not be possible to determine whether or not inhibition has occurred. In our previous studies phenol red (25) was used for this purpose.

In the present paper we wish to present studies in which the question of acid inhibition is reinvestigated, using a 2 per cent Liebig's extract test meal (26) as a stimulant instead of histamine because of the different mechanisms concerned in the action of these stimulants.

METHODS. The preparation and use of the 2 per cent Liebig's extract test meal has been described in detail (26) and will not be repeated.

In preparing the acid Liebig's extract test meal, a 4 per cent meal was first prepared and then diluted with equal parts of an acid twice the final strength desired.

In ashing the gastric samples for the total chloride determination it was necessary to reduce the amount of saturated sodium nitrate added to facilitate ashing to 0.08 cc. in experiments with the acid test meals. The usual amount (0.3 cc.) was used for the standard test meal experiments.

The animals were given a light feeding of milk and Karo syrup approximately 24 hours before the experiment. Just before starting the experiment the stomach was lavaged with 300 cc. of the test meal to be used. The meal was introduced by stomach tube and samples removed every half-hour for two hours. The stomach was emptied when the last sample was taken.

RESULTS. Four normal dogs and four dogs in which the entire stomach was isolated from the intestine were used. On the normal dogs 30 experiments were performed with the standard Liebig's extract test meal and 42 experiments in which the Liebig's extract was dissolved in varying strengths of hydrochloric acid. The acid meals were well tolerated and vomiting or evidence of nausea were absent.

A. *Normal dogs.* A complete set of experiments performed on the same dog is shown in table 1. The first 6 experiments were made with the standard test meal which is slightly acid but which, for convenience, will be referred to as the aqueous meal. Following this, experiments are shown in which the Liebig's extract was dissolved in increasing strengths of hydrochloric acid. The extra acid chloride per 100 cc. of gastric contents is shown in column 7. This value shows that there is practically no evidence of inhibition with the 0.056 normal acid meal. With the 0.077 normal acid meal definite evidence of inhibition is seen which increases and may be practically complete with approximately 0.10 normal acid meals. Since the stomach empties slower as stronger acid meals are used (19, 27, 28) there is more meal in the stomach to dilute the secreted acid (col. 17), hence the values just mentioned (col. 7) may give an erroneous impression of inhibition due in reality to greater dilution. When the results are expressed as the milligrams of extra acid chloride per 100 cc. of secretion entering the stomach the factor of dilution is eliminated. As shown in column 10 these values show similar changes, thus showing that the slower emptying time is not responsible for the observed decrease.

Results similar to those shown in table 1 were obtained on three other dogs. Before concluding that these findings represent true inhibition of acid secretion it is necessary to eliminate three other causes for the results. These are as follows:

1. *That the acid destroys the secretagogue in the Liebig extract.* To test this factor 2 per cent Liebig's extract in tenth normal hydrochloric acid was allowed to stand for varying periods up to ten days. It was then carefully neutralized with saturated sodium hydroxide until the acidity was as low or lower than that in the standard meal. These neutralized meals were found to be fully as potent as freshly prepared standard meals. The last experiment in table 1 illustrates this point. From this it appears unlikely that destruction of the secretagogue can explain the results.

2. *That when acid meals are placed in the stomach there is an increased regurgitation of duodenal secretions into the stomach, the acid secretion continuing unchanged. The increased amount of duodenal secretions, by dilution and neutralization, would lower the extra acid chloride per 100 cc. of gastric contents (col. 7) and likewise lower the acid chloride concentration of the total secretions entering the stomach (col. 10).* The decrease in the per cent of phenol red in the gastric samples (col. 4) shows the total amount of fluid entering the stomach. Examination of this figure shows that the total amount of fluid (secretions) entering the stomach with the acid test meals is in general *less* than with the standard aqueous meal. Thus it would be difficult to justify the above hypothesis. We endeavored to obtain a more quantitative expression of this fact. The method employed was as follows: Average curves for the total fluid, extra or non acid fluid, and the acid fluid were prepared from all experiments on each dog with the standard aqueous test meal and from all experiments on each dog in which the tenth normal acid meal was used. The average curves for each dog were then plotted on standard coördinate paper as shown in the upper right hand corner of figure 1. The areas under the curves are proportional to the amounts of the various fluids. The areas were determined by means of a standardized planimeter, and converted into terms of cubic centimeters of the various secretions. The illustration in figure 1 is typical of the results obtained. In table 2 the results obtained on four normal dogs are shown. It should be remembered that the values given represent the amounts of the various fluid secretions per 100 cc. of gastric contents, not the total amounts. The following points are important: 1. The total fluid (secretions) entering the stomach is always markedly less with the tenth normal acid Liebig's extract meal than with the standard aqueous meal. 2. The extra or non acid fluid (which includes the regurgitated duodenal secretions) shows small irregular changes, either remaining the same, increasing slightly or decreasing. 3. The acid fluid always shows marked reduction. Hence the reduction of the total fluid entering the stomach is due to reduction of the acid fluid. 4. The per cent of acid fluid in the total fluid entering the stomach is always markedly reduced, thus showing the results to be independent of the greater volume of meal in the stomach with the acid test meals, due to the slower emptying time. These results are sufficient to show that the

TABLE 1

*A complete series of experiments on dog II*

| COMPOSITION OF TEST MEAL, MGM. CHLORIDE PER 100 CC. | GASTRIC SAMPLE: Total chloride, mgm. per 100 cc. | GASTRIC SAMPLE: Neutral chloride, mgm. per 100 cc. | GASTRIC SAMPLE: P.S.P., per cent | EXTRA TOTAL CHLORIDE | EXTRA NEUTRAL CHLORIDE | EXTRA ACID CHLORIDE | CHLORIDE CONCENTRATION OF GASTRIC SECRETION: Total | CHLORIDE CONCENTRATION OF GASTRIC SECRETION: Neutral | CHLORIDE CONCENTRATION OF GASTRIC SECRETION: Acid | TOTAL FLUID | ACID FLUID | EXTRA FLUID | NEUTRAL CHLORIDE / EXTRA FLUID | BILE | TIME, MINUTES | VOLUME, CC. | REMARKS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Total..... 227 | 248 | 179 | 88 | 49 | 17 | 32 | 408 | 142 | 266 | 12 | 5 | 7 | 2.4 | 0 | 30 | 33 | |
| Neutral... 184 | 326 | 170 | 62 | 185 | 56 | 129 | 487 | 147 | 340 | 38 | 22 | 16 | 3.5 | 0 | 60 | 33 | |
| Acid..... 43 | 400 | 210 | 40 | 309 | 136 | 173 | 515 | 226 | 289 | 60 | 29 | 31 | 4.4 | + | 90 | 8 | |
| Total..... 227 | 254 | 191 | 91 | 47 | 24 | 23 | 522 | 267 | 255 | 9 | 4 | 5 | 4.8 | 0 | 30 | 32 | |
| Neutral... 184 | 348 | 174 | 52 | 230 | 78 | 152 | 479 | 163 | 316 | 48 | 25 | 23 | 3.4 | trace | 60 | 20 | |
| Acid..... 43 | 420 | 173 | 28 | 356 | 121 | 235 | 494 | 168 | 326 | 72 | 39 | 33 | 3.7 | ++ | 90 | 14 | |
| Total..... 224 | 246 | 200 | 93 | 38 | 24 | 14 | 543 | 344 | 199 | 7 | 2 | 5 | 4.8 | 0 | 30 | 36 | |
| Neutral... 189 | 284 | 191 | 67 | 134 | 64 | 70 | 406 | 194 | 212 | 33 | 12 | 21 | 3.1 | ++ | 60 | 33 | |
| Acid..... 35 | 248 | 164 | 34 | 172 | 100 | 72 | 261 | 151 | 110 | 66 | 12 | 54 | 1.9 | + | 90 | 33 | |
| | 346 | 140 | 26 | 288 | 91 | 197 | 389 | 123 | 266 | 74 | 33 | 41 | 2.2 | ++ | 120 | 33 | Standard 2% Liebig's extract test meal |
| Total..... 219 | 231 | 177 | 89 | 36 | 20 | 16 | 327 | 182 | 145 | 11 | 3 | 8 | 2.5 | 0 | 30 | 30 | |
| Neutral... 176 | 288 | 182 | 72 | 130 | 55 | 75 | 464 | 197 | 267 | 28 | 13 | 15 | 3.7 | ++ | 60 | 34 | |
| Acid..... 43 | 368 | 214 | 28 | 307 | 165 | 142 | 426 | 229 | 197 | 72 | 24 | 48 | 3.4 | ++ | 90 | 12 | |
| Total..... 220 | 246 | 173 | 94 | 39 | 4 | 35 | 650 | 66 | 584 | 6 | 6 | 0 | 0.0 | 0 | 30 | 30 | |
| Neutral... 180 | 306 | 183 | 76 | 139 | 46 | 93 | 580 | 192 | 388 | 24 | 16 | 8 | 5.7 | +++ | 60 | 35 | |
| Acid..... 40 | 360 | 177 | 55 | 239 | 78 | 161 | 531 | 174 | 357 | 45 | 27 | 18 | 4.3 | ++ | 90 | 30 | |
| Total..... 219 | 246 | 186 | 86 | 58 | 33 | 25 | 414 | 236 | 178 | 14 | 4 | 10 | 3.3 | trace | 30 | 29 | |
| Neutral... 178 | 312 | 186 | 74 | 150 | 54 | 96 | 577 | 208 | 369 | 26 | 16 | 10 | 5.4 | ++ | 60 | 31 | |
| Acid..... 41 | 384 | 266 | 66 | 239 | 149 | 90 | 705 | 438 | 265 | 34 | 15 | 19 | 7.8 | ++++ | 90 | 17 | |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Total..... 386 | 386 | 201 | 91 | 35 | 32 | +3 | 388 | 356 | 32 | 9 | 0.5 | 8.5 | 3.8 | + | 30 | 30 | 2% Liebig's extract test meal in 0.56 normal hydrochloric acid |
| Neutral... 186 | 402 | 209 | 85 | 74 | 51 | +23 | 493 | 340 | 153 | 15 | 4.0 | 11.0 | 4.6 | ++ | 60 | 30 | |
| Acid..... 200 | 410 | 178 | 62 | 171 | 63 | +108 | 450 | 166 | 284 | 38 | 18.0 | 20.0 | 3.2 | ++ | 90 | 25 | |
| | 442 | 186 | 54 | 234 | 86 | +148 | 509 | 187 | 322 | 46 | 25.0 | 21.0 | 4.1 | +++ | 120 | 32 | |
| Total..... 446 | 438 | 177 | 91 | 32 | 20 | +12 | 356 | 223 | 133 | 9 | 2.0 | 7.0 | 2.9 | trace | 30 | 31 | Same in 0.077 normal hydrochloric acid |
| Neutral... 172 | 432 | 192 | 85 | 53 | 46 | +7 | 353 | 307 | 46 | 15 | 1.0 | 14.0 | 3.3 | + | 60 | 31 | |
| Acid..... 274 | 420 | 210 | 76 | 81 | 79 | +2 | 337 | 329 | 8 | 24 | 0.3 | 23.7 | 3.3 | ++ | 90 | 95 | |
| Total..... 492 | 482 | 177 | 96 | 10 | 16 | −6 | 250 | 250 | 0 | 4 | 0 | 4 | 4.0 | 0 | 30 | 30 | Same in 0.091 normal hydrochloric acid |
| Neutral... 168 | 474 | 174 | 88 | 41 | 26 | +15 | 342 | 216 | 126 | 12 | 3 | 9 | 2.9 | + | 60 | 30 | |
| Acid..... 324 | 466 | 191 | 83 | 58 | 52 | +6 | 341 | 306 | 35 | 17 | 1 | 16 | 3.3 | ++ | 90 | 30 | |
| | 460 | 227 | 75 | 91 | 101 | −10 | 364 | 364 | 0 | 25 | 0 | 25 | 4.0 | +++ | 120 | 73 | |
| Total..... 552 | 536 | 197 | 94 | 17 | 20 | −3 | 284 | 284 | 0 | 6 | 0 | 6 | 3.3 | 0 | 30 | 30 | Same in 0.102 normal hydrochloric acid |
| Neutral... 188 | 522 | 201 | 88 | 36 | 36 | 0 | 300 | 300 | 0 | 12 | 0 | 12 | 3.0 | 0 | 60 | 29 | |
| Acid..... 364 | 498 | 227 | 79 | 62 | 78 | −16 | 295 | 295 | 0 | 21 | 0 | 21 | 3.7 | trace | 90 | 30 | |
| | 474 | 245 | 76 | 54 | 102 | −48 | 225 | 225 | 0 | 24 | 0 | 24 | 4.3 | + | 120 | 95 | |
| Total..... 548 | 540 | 186 | 94 | 25 | 21 | +4 | 416 | 350 | 66 | 6 | 0.7 | 5.3 | 4.0 | 0 | 30 | 36 | Same in 0.105 normal hydrochloric acid |
| Neutral... 176 | 524 | 191 | 91 | 26 | 31 | −5 | 289 | 289 | 0 | 9 | 0.0 | 9.0 | 3.5 | 0 | 60 | 35 | |
| Acid..... 372 | 516 | 200 | 83 | 61 | 54 | +7 | 359 | 318 | 41 | 17 | 1.0 | 16.0 | 3.4 | trace | 90 | 35 | |
| | 492 | 207 | 76 | 76 | 73 | +3 | 316 | 304 | 12 | 24 | 0.5 | 23.5 | 3.1 | + | 120 | 104 | |
| Total..... 223 | 354 | 159 | 58 | 225 | 57 | +168 | 535 | 135 | 400 | 42 | 28 | 14 | 4.1 | + | 55 | 67 | Standard 2% meal followed by 2% meal in 0.103 normal hydrochloric acid |
| Neutral... 176 | | | | | | | | | | | | | | | | | |
| Acid..... 47 | | | | | | | | | | | | | | | | | |
| Total..... 544 | 516 | 174 | 94 | 5 | 7 | −2 | 83 | 83 | 0 | 6 | 0 | 6 | 1.2 | trace | 30 | 34 | |
| Neutral... 178 | 508 | 197 | 88 | 29 | 40 | −11 | 242 | 242 | 0 | 12 | 0 | 12 | 3.3 | + | 60 | 35 | |
| Acid..... 366 | 492 | 204 | 83 | 41 | 56 | −15 | 241 | 241 | 0 | 17 | 0 | 17 | 3.3 | + | 90 | 131 | |
| Total..... 550 | 512 | 192 | 83 | 56 | 34 | +22 | 329 | 200 | 129 | 17 | 4 | 13 | 2.6 | 0 | 90 | 120 | 2% meal in 0.101 normal hydrochloric acid followed by a standard 2% meal |
| Neutral... 190 | | | | | | | | | | | | | | | | | |
| Acid..... 360 | | | | | | | | | | | | | | | | | |
| Total..... 226 | 270 | 168 | 83 | 82 | 24 | +58 | 482 | 141 | 341 | 17 | 10 | 7 | 3.4 | trace | 30 | 35 | |
| Neutral... 173 | 356 | 150 | 55 | 232 | 55 | +177 | 516 | 122 | 394 | 45 | 30 | 15 | 3.7 | trace | 60 | 86 | |
| Acid..... 53 | | | | | | | | | | | | | | | | | |
| Total..... 552 | 552 | 510 | 93 | 38 | 12 | +26 | 543 | 172 | 371 | 7 | 4 | 3 | 4.0 | 0 | 30 | 29 | 2% test meal in 0.102 normal hydrochloric acid neutralized with saturated NaOH |
| Neutral... 536 | 548 | 378 | 63 | 200 | 40 | +160 | 540 | 108 | 432 | 37 | 27 | 10 | 4.0 | + | 60 | 30 | |
| Acid..... 16 | 528 | 352 | 38 | 318 | 149 | +169 | 513 | 240 | 273 | 62 | 28 | 34 | 4.4 | + | 90 | 18 | |

changes observed are not dependent upon increased duodenal regurgitation. They likewise show that the reduction in acid fluid is not accompanied by an equivalent increase in non acid fluid.

3. *That when an acid deficit occurs it is due to absorption of acid from the stomach and that acid absorption may explain the apparent inhibition.* In analyzing this factor only those samples which showed an acid deficit

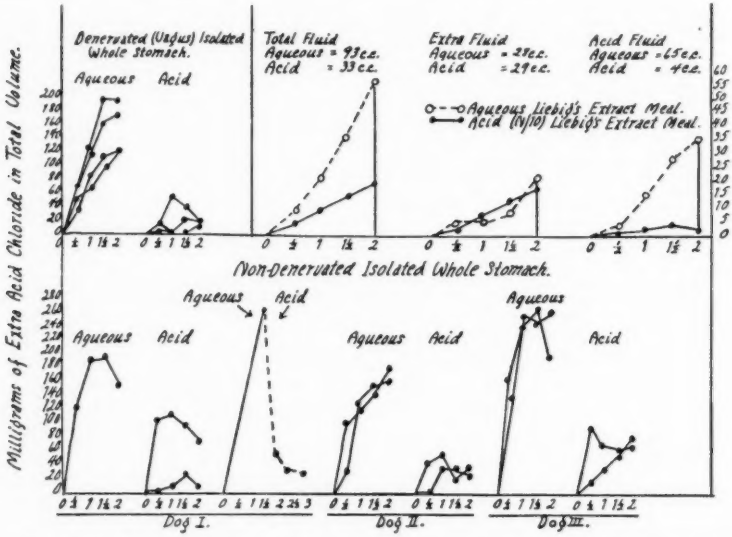


Fig. 1. *1. Lower half and upper half left hand corner.* Experiments on non denervated and partially vagotomized whole stomachs isolated from the intestine. The aqueous Liebig's extract test meal and the tenth normal acid Liebig's extract test meal were used on each dog. The 3rd experiment on dog I is one in which the aqueous test meal was followed immediately by the tenth normal acid test meal.

*2. Upper right hand corner.* Average curves for all experiments with the aqueous Liebig's extract test meal and the tenth normal acid Liebig's extract test meal on dog I. The areas under the respective curves were determined with a standardized planimeter and converted into cubic centimeters of the various fluids. The results represent the amounts of the various secretions per 100 cc. of gastric contents, not the total amounts. A similar analysis of all experiments on four dogs is presented in table 2.

(33 in number) were used. The *total acid deficit* is the difference between the acid chloride concentration of the test meal and that of the gastric sample. In figure 2 (left half) it is seen that as the total acid deficit increases there is also an increase in the total fluid entering the stomach, the total neutral chloride, the extra neutral chloride and the chloride (neutral) of neutralized acid. The total neutral chloride is composed of the neutral

chloride of the neutralized acid plus the *extra* neutral chloride which is the neutral chloride of the non acid fluids of intragastric and duodenal origin. The curve of the chloride of neutralized acid is seen to agree very well with the theoretical curve based on previous experiments (29) in which it was shown that approximately one-third of the total neutral chloride represents neutralized acid. The agreement between the observed and theoretical values for the chloride of neutralized acid plus the concomitant increase in the five factors enumerated above make it very unlikely that absorption of acid is a factor in producing the acid deficits.

TABLE 2

*An analysis of the average secretion in experiments with the aqueous Liebig's extract test meal and the tenth normal acid Liebig's extract test meal*

Results obtained as explained in text and in figure 2

| GASTRIC SECRETION | AQUEOUS LIEBIG'S EXTRACT TEST MEAL | N/10 ACID LIEBIG'S EXTRACT TEST MEAL | DOG |
|---|---|---|---|
| Total fluid, cc. | 93 | 33 | I |
| Acid fluid, cc. | 65 | 4 | |
| Extra fluid, cc. | 28 | 29 | |
| Per cent acid fluid | 70 | 12 | |
| Total fluid, cc. | 69 | 29 | II |
| Acid fluid, cc. | 34 | 1 | |
| Extra fluid, cc. | 35 | 28 | |
| Per cent acid fluid | 49 | 3.5 | |
| Total fluid, cc. | 38 | 28 | III |
| Acid fluid, cc. | 18 | 0 | |
| Extra fluid, cc. | 20 | 28 | |
| Per cent acid fluid | 47 | 0 | |
| Total fluid, cc. | 91 | 40 | IV |
| Acid fluid, cc. | 62 | 8 | |
| Extra fluid, cc. | 29 | 32 | |
| Per cent acid fluid | 68 | 8 | |

They are due to the fact that since no acid is being secreted by the stomach, the non acid fluids of the stomach and duodenum lower the acidity of the test meal by neutralization.

The above analyses are sufficient to show that the results are due to true inhibition of acid secretion.

*The composition of the secretions entering the stomach in experiments with aqueous and acid Liebig's extract test meals.* In a previous publication (19) it was shown that the chloride concentration of the secretion of fundic pouches, stimulated with histamine, is the same whether the pouch is

filled with distilled water, 0.10 normal or 0.173 normal hydrochloric acid solutions. It is therefore important to know whether the composition of various secretions entering the stomach is the same with the acid and aqueous Liebig's extract test meals. An analysis of the experiments performed on four normal dogs is shown in the right half of figure 2. In making this analysis we *assumed* that the concentration of the secreted acid was the same in both (600 mgm. of acid chloride per 100 cc.). All samples from

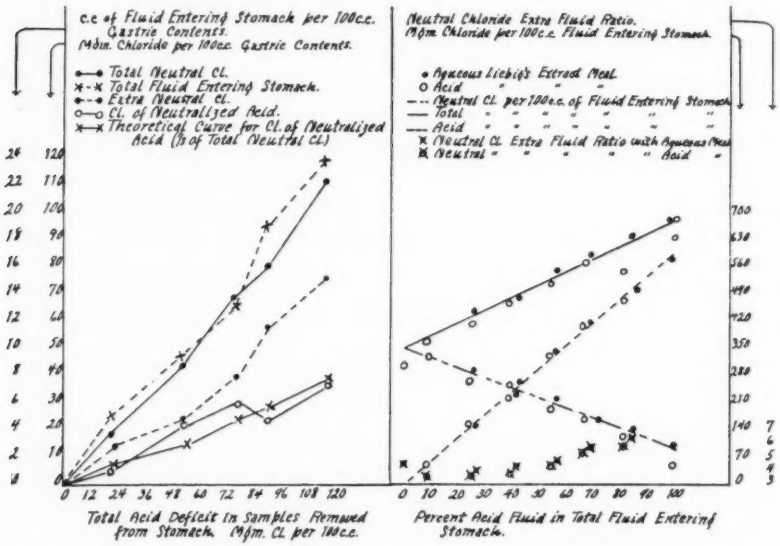


Fig. 2. *1. Left half.* An analysis of 33 samples from experiments with the tenth normal acid Liebig's extract test meal in which acid deficits occurred. The experiments were grouped according to increasing acid deficits and the average values for the various constituents in each group determined.

*2. Right half.* An analysis of experiments performed on four normal dogs with the aqueous Liebig's extract test meal and Liebig's extract in 0.056, 0.077 and 0.10 normal hydrochloric acid. The aqueous experiments consisted of 123 half-hour samples and the acid experiments of 111 half-hour samples. It is seen that the average values for the various constituents of the secretion are the same in the aqueous and acid test meal experiments.

both sets of experiments were then grouped according to increasing percentages of acid fluid in the total fluid entering the stomach. It was then found that the *total* and *neutral* chloride concentrations of the total fluid as well as the neutral chloride-extra fluid ratios were practically the same in both sets of experiments. This fact indicates that the acidity of the gastric contents does not alter the strength of the acid secreted, it merely influences the amount secreted.

In the experiments in which an acid deficit occurred (33 in number) it was possible to determine the composition of the non acid secretions. It was found that the neutral chloride content averaged 302 mgm. per 100 cc. The average alkalinity in all experiments was 0.044 normal, in individual dogs the average varied from 0.02 to 0.05 normal. Further analysis showed that the lowering of the acidity of the test meal was due primarily to dilution (76 per cent) and secondarily to neutralization (24 per cent). It was also found that only 34 per cent of the neutral chloride represented neutralized acid while 66 per cent was a constituent of the non acid fluids of intragastric and duodenal origin. These values are all in excellent agreement with those previously reported (20–31).

B. *Studies on the whole stomach isolated from the intestine.* The above studies show that when the acidity of the gastric contents reaches a certain level, inhibition of acid secretion occurs. The next question is whether this inhibitory effect operates through intragastric or duodenal mechanisms. To settle this question four isolated whole stomachs were studied. In three of these the nerve supply (both vagus and sympathetic) was intact, the stomach was separated from the intestine but attached to the esophagus and opened to the outside by a gastrostomy. In one the stomach was partially denervated (vagus) separated from the intestine and opened to the surface by a gastrostomy. The esophagus was anastomosed to the duodenum. There was very little difference in the response of the partially and non-denervated preparations.

The general method of performing the experiments was as follows: Accumulated secretions were removed and the stomach thoroughly lavaged 3 or 4 times with 200 cc. portions of the test meal to be used. The test meal was then introduced and samples removed every half-hour for two hours. The amount removed each half-hour was approximately the same and was sufficient to empty the stomach in two hours.

Several experiments were performed on each animal with the aqueous and tenth normal hydrochloric acid Liebig's extract meal. The results are shown in the lower half and upper left hand corner of figure 1. The results are expressed as *total amount* of extra acid chloride present in the entire gastric sample not as milligrams per 100 cc. It is seen that there was always a marked reduction in the amount of acid secreted when the tenth normal acid meal was used, but only very rarely was the secretion completely inhibited. The cause of this residual secretion is not clear. It was not due to distention of the stomach since reduction in the volume of meal used from 250 or 300 cc. to 100 cc. caused practically no change. Regardless of this the results show quite clearly that inhibition by acid gastric contents may be an intragastric mechanism independent of reflex or hormone influences from the duodenum.

*The rapidity of onset and duration of the inhibitory effect.* This was studied in the four normal dogs and in the isolated stomachs (table 1 and

fig. 1). *The rapidity of onset* of acid inhibition was studied by first introducing the aqueous Liebig's extract meal and allowing it to remain long enough (1 to 1½ hours) to stimulate a high rate of secretion. The aqueous meal was then removed and the stomach lavaged with 300 cc. of the tenth normal acid Liebig's extract meal. The acid meal was then introduced and samples removed every half-hour. The results show that the inhibitory effect begins immediately in spite of the previous high rate of secretion. Identical results were obtained in the intact and isolated stomach. An analysis of these experiments indicates that the stomach is actually more susceptible to acid inhibition when the secretion rate is high than when the stomach is resting. *The duration of the inhibitory effect* was studied by reversing the procedure. The acid meal was first introduced, allowed to remain for 1½ hours and removed. The stomach was then lavaged with a portion of the aqueous meal, the meal introduced and samples removed every half-hour. The results show that there is no hangover of the inhibitory effect, it apparently terminates when the acid meal is removed.

Discussion. The present studies show that when a Liebig extract meal is used, the presence of acid in the stomach, above a certain minimal concentration, causes a progressive inhibition of acid secretion. The difference between these and our previous studies using histamine stimulation (19) is important and shows that histamine is either qualitatively or quantitatively different from the stimulation caused by meal extract. This difference shows that histamine should not be used when normal regulatory mechanisms are to be studied.

When a tenth normal hydrochloric acid Liebig's extract meal was neutralized it was found that the inhibitory properties were removed. Since the total chloride concentration was practically the same before and after neutralization, it follows that the inhibitory effect is due to the hydrogen and not to the chloride ion.

Practically no inhibitory effect was noted until the strength of the acid in the test meal was above 0.06 normal. It is possibly significant to recall that clinical observers have long maintained that 0.06 normal or 60 clinical units is approximately the normal, maximal value for the total acidity of the gastric contents during a test meal.

The degree of inhibition varies in different dogs. The tenth normal acid meal may completely inhibit some animals while others show a definite but much less marked effect. In general those animals which show a low rate of acid secretion with the aqueous Liebig's extract meal are well inhibited while those with a high rate are not, although this relationship is not invariable.

Since the composition of the acid secretion was not altered when acid inhibition occurred it follows that changes in the strength of the acid secreted is not one of the mechanisms normally regulating gastric acidity.

The experiments with the isolated whole stomachs show that acid inhibi-

tion is an intragastric phenomenon which does not depend on either reflex or hormone influences from the intestine. Thus enterogastrone (Ivy) is not involved in the process. That inhibitory effects may, under certain conditions, have their origin in the intestine is not excluded or denied.

The rapidity of onset of acid inhibition when the secretion rate of the stomach is high and its abrupt termination when the acid meal is withdrawn from the stomach quite definitely exclude a hormone or humoral mechanism. Kim and Ivy (32) have concluded that secretogogues in the stomach act not by being absorbed but by a hormone or nervous mechanism. The rapidity with which secretogogue action is inhibited by acid suggests that a nervous mechanism is involved in secretogogue action in the stomach.

MacLean (3, 4, 5) and his co-workers in their studies on acid inhibition proposed the theory that during inhibition there is simply a failure to convert neutral chloride to acid chloride, hence the total chloride concentration and the total amount of secretion remain the same, the secretion being converted into a non-acid diluting fluid. Our results are not in agreement with this theory since we find that the total chloride concentration and the total fluid secreted both decrease while the amount of non acid fluid remains practically unchanged.

Just what rôle acid inhibition may play in the normal regulation of gastric acidity is difficult to say. In the intact stomach the normal regurgitation of duodenal contents, just before the stomach empties (26) makes it difficult to separate the effects of the duodenal secretions from the possible effects of acid inhibition. In the experiments on isolated whole stomachs in which the aqueous meal was used (fig. 1), it is seen that the increase in acid chloride becomes progressively smaller or may stop as the acidity of the contents rises. Since duodenal regurgitation is excluded, this behavior may possibly be the result of acid inhibition.

It is important to note that the inhibitory effects observed in the present experiments are probably due almost entirely to inhibition of the gastric phases of secretion. This is obvious first, because the mode of administering the test meal (stomach tube or gastrostomy) excluded the psychic phase, and second, because the experiments lasted only two hours and were thus completed before the intestinal phase could have become definitely apparent (32). Whether or not acid will inhibit the intestinal and psychic phases cannot be stated at present.

## SUMMARY

1. Using a two per cent Liebig's extract test meal it was found that when the Liebig's extract was dissolved in hydrochloric acid solutions there was a progressive inhibition of acid secretion as the strength of the acid was increased. No inhibition occurred until the acidity was above 0.056 normal but it was often nearly complete when 0.10 normal acid solutions were used. It was shown that the results are due to true inhibition of acid

secretion and not to destruction of secretogogue in the Liebig's extract, increased duodenal regurgitation or absorption of acid from the stomach.

2. The inhibitory phenomenon is of intragastric origin since it occurs in the stomach isolated from the intestine.

3. The composition of the acid and non-acid fluids entering the stomach is not altered when acid inhibition occurs. A reduction in the secretion of acid fluid is the only change which occurs.

4. Evidence is presented which suggests that the inhibition is not due to hormone action.

5. The inhibition noted in the present studies is possibly largely or solely due to inhibition of the gastric phases of secretion, since the cephalic and intestinal phases are probably not active under the conditions of the experiments.

## REFERENCES


(1) Pavlov, I. P. The work of the digestive glands. Charles Griffin & Co. London 1910, p. 115.
(2) Roseman, R. Pflüger's Arch. **118:** 467, 1907.
(3) MacLean, H., W. J. Griffiths and B. W. Williams. J. Physiol. **65:** 77, 1928.
(4) MacLean, H. and W. J. Griffiths. J. Physiol. **66:** 356, 1928.
(5) MacLean, H. and W. J. Griffiths. J. Physiol. **65:** 63, 1928.
(6) Apperly, F. L. Brit. J. Exper. Path. **7:** 111, 1926.
(7) Apperly, F. L. and J. H. Norris. J. Physiol. **70:** 158, 1930.
(8) Goldberg, S. L. Arch. Int. Med. **49:** 816, 1932.
(9) Carlson, A. J. Physiol. Reviews **3:** 1, 1923.
(10) Farrell, J. I. This Journal **85:** 672, 1928.
(11) Vandorfy, J. Med. Klinik **20:** 997, 1924.
(12) Paul, W. D. Personal communication.
(13) Webster, D. R. This Journal **90:** 718, 1929.
(14) Bolton, C. and G. W. Goodhart. J. Physiol. **77:** 287, 1933.
(15) Bolton, C. and G. W. Goodhart. J. Physiol. **73:** 8, 1931.
(16) Hollander, F. and G. R. Cowgill. This Journal **91:** 151, 1931.
(17) Hollander, F. This Journal **98:** 551, 1932.
(18) Hollander, F. J. Biol. Chem. **97:** 585, 1932.
(19) Wilhelmj, C. M., I. Neigus and F. C. Hill. This Journal **106:** 381, 1933.
(20) Wilhelmj, C. M., I. Neigus and F. C. Hill. This Journal **107:** 490, 1934.
(21) Wilhelmj, C. M., L. C. Henrich, I. Neigus and F. C. Hill. This Journal **108:** 197, 1934.
(22) Wilhelmj, C. M., L. C. Henrich and F. C. Hill. This Journal **110:** 251, 1934.
(23) Wilhelmj, C. M., L. C. Henrich and F. C. Hill. This Journal **111:** 293, 1935.
(24) Hill, F. C., L. C. Henrich and C. M. Wilhelmj. Arch. Surgery **31:** 622, 1935.
(25) Gorham, F. D. J. A. M. A. **81:** 1738, 1923.
(26) Wilhelmj, C. M., F. T. O'Brien and F. C. Hill. This Journal (in press).
(27) Stewart, J. L. and W. N. Boldyreff. This Journal **102:** 276, 1932.
(28) Thomas, J. E. J. A. M. A. **97:** 1663, 1931.
(29) Wilhelmj, C. M., L. C. Henrich, I. Neigus and F. C. Hill. This Journal **112:** 15, 1935.
(30) Wilhelmj, C. M., L. C. Henrich and F. C. Hill. Proc. Soc. Exper. Biol. and Med. **31:** 969, 1934.
(31) Hill, F. C., L. C. Henrich and C. M. Wilhelmj. Surg. Gynec. and Obst. **60:** 1, 1935.
(32) Kim, M. S. and A. C. Ivy. This Journal **105:** 220, 1933.

# THE LATENCIES OF MECHANICAL AND ELECTRICAL RESPONSES IN SKELETAL MUSCLE[1]

CHARLES D. SNYDER

*From The Department of Physiology of The Johns Hopkins University School of Medicine*

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It was perhaps the work of Einthoven and his associates that most of all reopened the problem of muscle latencies. Their work at first was on heart muscle but finally Roos (1932) published a paper on frog's skeletal muscle in which he contended that the onset of mechanical response in skeletal muscle, as his associates had contended for cardiac muscle, is instantaneous with the onset of electrical response. Roos publishes very few of his observations, and bases his argument not on a statistical datum but apparently upon a few readings, that he considers crucial, and upon theoretical grounds. He concludes that an observed mechanical latency longer than that of the electric response is due to inadequacies of the recording devices used, and should be disregarded. The $0.4\sigma$, the least latency he observed for change of form, he argues is due to the inability one has in reading correctly the actual beginning rise of the lever record from its base line.

The final test then revolves upon the specifications and construction of the apparatus used and the method adopted to pick up the earliest phase of the mechanical effect of the muscle's action. Not only must the mass of the lever system and the tension it exerts on the relaxed muscle be carefully chosen but the natural vibration period of the lever arms must be small enough to enable them to respond with adequate promptness.

To meet the difficulty of reading the moment of rise from the base-line it occurred to me that if one varied the speed of the registration surface to correspond to variation in rate of tension development in the muscle (caused by change of temperature), the initial curves of the various mechanograms ought to be well nigh superposable. The errors then would tend more nearly to be equalized, especially in case the number of readings at each temperature was not the same. An illustration of records thus obtained at different temperatures is shown in figure 1, part A. In all three

[1] Mr. David Lubin, *cand. med.*, collaborating with the author during the preliminary stages of this investigation, designed, built and tested much of the accessory apparatus.

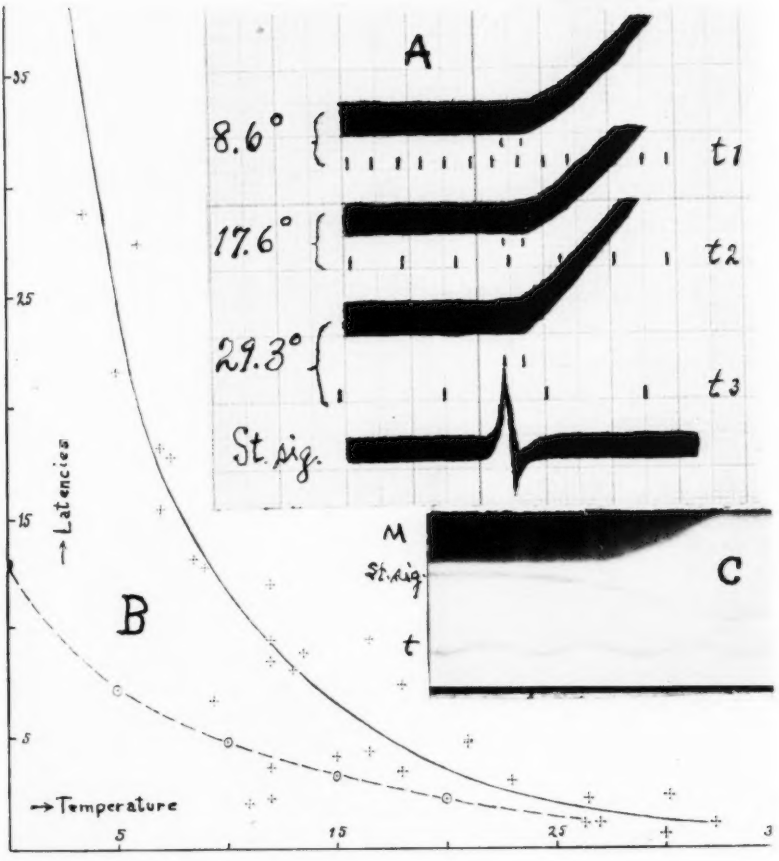


Fig. 1. A illustrates method of timing movement of film to rate of tension development or to the latency of muscle. The records are read from left to right. The three mechanograms are of the muscle at the temperatures indicated and their corresponding film speeds are indicated as *t1*, *t2* and *t3*, which show that 0.01 second covers nearly 3.5, 8 and 15 mm. distance respectively in the three photographs. The electric stimulating signal appears only once, below, *St. sig.* It will be noted that the latent periods of all three mechanograms are nearly 3 mm. of linear space, but that these 3 mm. have time values varying as their respective time traces (0.01″) immediately below indicate. The tracings are mirror images from three original photographs reproduced on millimeter paper over a light-box, with their respective moments of stimulation (apex of thread deflection, and then the rest of the record) all brought to the same coördinate.

B. The data plotted in crosses (+, +, +) and the continuous curved line drawn through them, are the "corrected" latencies of thickening of the muscles. The data plotted in circles (○, ○, ○) and the broken line connecting them are taken from a smoothed curve constructed from Samojloff's observations on motor end-plate delay. The ordinates are given in milli-seconds, the abscissae in degrees Centigrade.

C illustrates a record of muscle thickening with electro-magnetic stimulus signal. Temperature of muscle 20°C., time intervals, 01 second.

cases delay from peak of first deflection of the galvanometer stimulus-signal to onset of lever-rise is nearly 3 mm. of film; but the time value of unit distance varies nearly as much as 1:2:4.

But aside from these technical difficulties, and as will be pointed out later on in the discussion, there are good reasons on theoretical grounds still to entertain the view that the latent period of muscle is real. A reinvestigation, therefore, seemed to be demanded.

EXPERIMENTAL. In all the experiments the gastrocnemius of *R. pipiens* was used. Single, just maximal break induction shocks were applied directly to the muscle.[2] The experiments were done in two series; one recording only the movement due to thickening of the muscle, the other recording only the movement due to shortening, or to tension development.

In order to have the change of temperature affect the muscle only, rather than also the recording apparatus, specially devised small glass-chambers, such as I used in a former research (1912), were blown with double walls. Water of any desired temperature could be made to circulate between the walls and thus only affect the muscle, together with the metal stimulating electrodes and in addition, in the experiments on thickening response, one arm only of the muscle lever.

The chamber designed for the recording of muscle thickening (fig. 2) was made large enough to admit, besides the muscle, the bulb-end and about 5 cm. of the stem of a small thermometer whose reading scale extended outside the chamber. The thermometer was attached to a pair of metal rails upon which, and around the edges of the mercury bulb, was built a small platform of paraffin with the electrodes so imbedded and disposed that the muscle, when laid horizontally thereon, came in contact below with one stimulating electrode on a line with its greatest diameter (neural equator), and above with the other electrode at the proximal end of the Achilles tendon. The electrodes were of platinum; the metal rails served as leads to the outside. The leads, electrodes, muscle-platform and thermometer were thus built into a unit which, with muscle mounted in position, could be easily slipped into the muscle chamber and sealed up, and again unsealed and removed as desired. Preparatory to the latter operations the muscle-lever could be lifted up out of the way by its external arm.

The muscle lever (see fig. 2) was erected on a thin lead plate somewhat curved to fit the muscle. On the upper surface of this plate was soldered one side of a small tetrahedron fashioned on fine wire, from the free apex of which the wire was extended upward as the recording lever-arm. The latter was passed through a fine aperture provided in the double-walled

[2] The latent period of this muscle is shortest 1, when the negative pole of the stimulating current is the one nearer the neural equator (S. Zeldendrust, 1933), and 2, when just maximal shocks, evoking maximal tensions are used (Furnhjelm, Hortling and Renquist, 1934).

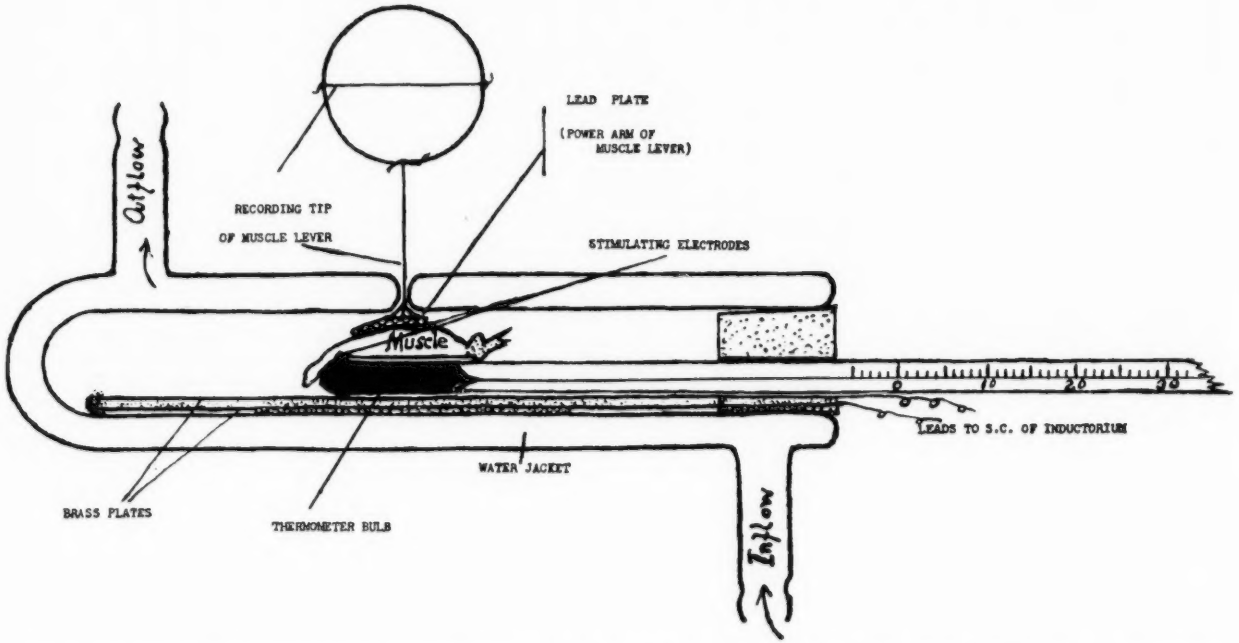


Fig. 2. The muscle chamber and muscle lever used to record thickening response, shown in longitudinal section, in original dimensions. The brass-plates are half-cylinders in shape; each is in contact with a stimulating electrode, and properly insulated,—all of which is not shown in the drawing. A beam of light was focused on the "recording tip," at ⌊s in its path to the photographic recording apparatus. See text for further details.

muscle-chamber just above the point where the muscle lay when sealed in position in the chamber. This outer part of the lever was finally then further bent into a complete ring. Across the horizontal diameter of the ring was stretched a silver wire of 0.05 mm. diameter, to serve as the recording part of the lever. The muscle chamber then was mounted close to the objective of a compound microscope and the recording lever brought in position for projection upon a camera shutter. The ring and filament of the lever thus follow the suggestion of Einthoven-de Jongh for micro-photographic recording and as described for their wire-myograph. The lever of this latter instrument, on account of its greater dimensions, must have had a much lower vibration frequency than the one here described.

The lead-plate, having been first thoroughly but thinly coated with paraffin, was laid over the muscle so that one edge lay along the greatest diameter while the rest of the plate extended over the muscle distal thereto, ending with the corners of the other edge on the muscle-platform. When the muscle contracted, the thickening being greatest at the belly, the edge of the plate lying across the belly was tilted upward, the edge lying on the platform remaining stationary. The plate thus acted as one arm, the wire erected at right angles thus acted as the other arm of a lever. This latter arm being longer, magnified the movement; the micro-photographic projection effected further amplification to about 160 times. Further magnification it was found had no particular advantage. The objection to this lever as I used it was the element of friction that occurred at the aperature in the muscle-chamber; but the edges of the aperture were so smooth and the surface so small that I was convinced that this element was of a negligible quantity. Kleinknecht (1924) used a mirror and, contrary to Fulton's understanding of this (1926), had the whole of his lever inside the muscle chamber; temperature changes thus affected the whole of the lever system as well as the muscle, but the element of friction was avoided.

In the muscle chamber designed for recording changes of tension, or of length of the muscle, the thermometer was inserted into a side-tubule of the in-flow tube and none of the lever excepting the connecting thread extended to the inside of the chamber. The size of chamber therefore could be greatly reduced. The stimulating electrodes were so placed as to come in contact with the muscle in the same regions described above, only the electrode near the tendon was thinner and more flexible and was bent into a few spiral turns to allow the muscle to pull against its slight stretching tension. A short inelastic thread drawn through a fine aperture in the wall of the chamber connected muscle to lever. The lever was of the wire-myograph form of Einthoven and Hugenholtz, and had the following specifications. A fine piano wire 0.2 mm. in diameter was stretched and its ends clamped tightly between heavy brass jaws the inner edges of which were 15 mm. apart, which determined the tortional

vibrating length of the wire. Across the center of this wire was balanced and rigidly fastened edgewise a bit of fret-saw, 0.5 mm. thick, 14 mm. long and 2 mm. wide; the arm extending 7 mm. toward the holder, served for attaching the muscle; the arm extending out away from the holder served as attachment for the recording tip of stainless steel, 0.1 mm. diameter, whose extreme tip was made still thinner by hammering. The short arm of this lever was then placed a few millimeters above the top of the muscle chamber, and was connected to the muscle with silk thread of 3 cm. length. This arrangement permitted the tip of the recording arm to extend just half way across the focal place of the objective of the projection microscope. The natural period of this lever was 508 complete vibrations per second. The rate of damping was exceedingly rapid. Its tension values were calibrated in the usual way.

A calibrated tuning fork of 100 D.V. per second was used throughout the series of experiments. The method of recording the moment of stimulating the muscle was varied. During the earlier part of the work a contact key, on an electromagnet driven arm placed in the primary circuit of a Harvard inductorium, was automatically broken by the relay circuit that was operated by the revolving camera drum just after the camera shutter opened. There was a methodical error in this stimulus-signal that proved to have a lag of about $0.71\sigma$ later than the onset of deflection of a tight thread of a thread galvanometer when placed in parallel with the *primary* circuit of the inductorium. Later on, when the technique of photographing simultaneously two shadows from two projecting lanterns on one and the same film was perfected, the stimulating signal used was that of the thread of a thread-galvanometer put in parallel with the muscle in the *secondary* circuit of a coreless inductorium. The automatic break key of the primary circuit, in this case, was opened by the release mechanism and relay operated by the revolving drum of the camera.

The camera used was one designed for the Mathews oscillograph. By a modification of the retarding mechanism, speeds could be adjusted from *ca* 38 cm. to 155 cm. per second.

RESULTS AND THEIR TREATMENT. In evaluating the readings of latent period it will be necessary to correct for delays that can be attributed to any sources whatever other than those within the muscle-organ itself. In the present experiments these extrinsic factors are

a. The delay of the stimulus signal. This applies to the readings on muscle thickening only (fig. 1B) and was added as a constant, $0.71\sigma$, in every reading where this signal was used.

b. The delay of the excitation itself, or the effective duration of the stimulus. Since the duration of the stimulus was frequently recorded by a tight thread of a thread-galvanometer during experiments on thickening, and in every record of the shortening experiments its time can be stated,

fairly accurately, as lasting about $2\sigma$. Further the stimuli being always maximal break induced shocks applied directly to the muscle and of considerable absolute current strength, they doubtless had a time of utilization nearly that of chronaxie or even less. I estimate this time of utilization therefore to have been only a few tenths of a sigma at most. (Conf. van der Heyde and Wertheim Solomonson.) Now this time was not subtracted from the data plotted in figure 1B, for to subtract, say 0.1 to 0.4 sigma, along the curve from right to left would not change its character perceptibly. In reading the records on muscle shortening (table 2) the peak of the first galvanometer deflection was used as zero time. The time to peak having been about $2\sigma$ it is clear in these cases a good deal more than time of utilization must have been subtracted. We can be certain at least that for these records this factor needs no further consideration.

c. When muscles are not curarized and the direct stimulus is just maximal it is quite probable that the end-plates only are excited, and then finally the muscle by transfer of the end-plate excitation. For much stronger currents it is further probable that no end-plate excitation whatever is effective, all the muscle fibers receiving directly maximum shock at once. But in this case the fibers may become altered by excessively strong currents. With intermediate strengths some fibers may be stimulated directly others only through their end-plates. In the case end-plates are involved the delay of transference which according to Samojloff (1925) is considerable at room-temperature and increases greatly with decrease of temperature. By placing one lead on the neural equator and the other at the distal end of frogs' gastrocnemius muscle, this author observed a prodromal positive deflection. This he interpreted to be the action potential of the excitation wave passing along the motor end-plates, for it disappeared as soon as the proximal lead was placed below the neural equator. It has been argued that this prodromal deflection may have been derived from the fine branches of the sciatic nerve where they separate from the main trunk on their way to the motor-end plates. However one views the phenomenon, it is a delay that should be deducted from the gross delays observed in these experiments (see Fulton, 1926).

From the work of Lindhard (1932) and others there is a possibility that the end-plate delay as measured by Samojloff is too small. For if it is true as Lindhard maintains that the action potential heretofore ascribed to muscle is derived entirely from motor end-plates then at least the time to peak of the first negative deflection of the electric response would have to be included in this delay. However upon careful examination *a*, of my own records of muscle action potentials led off from the distal half of both normal and curarized gastrocnemius muscles of frog, and *b*, of the many published records of similar results I cannot admit that Lindhard's view is the correct one. The species of frog I used is different from that

used by Samojloff; but the great number of physiological durations of nerve processes that have been found to be the same in many species will justify us in taking over the data on end-plate delay. All the readings in this paper have been so corrected.

In consideration of all these facts it was decided, therefore, to deal only with muscles that had not been curarized and that responded to just maximal, direct stimulation, assuming that the stimulus used would affect only the motor-end-plates.

A word may here be said on the "period of rigidity." The tracings reproduced in figure 1, A, happen not to show this phenomenon, but all my records on muscle thickening did show it and many on muscle shortening, although probably not as sharply as in Fulton's records (see fig. 1, C). From my readings, however, I cannot see that the presence of a period of rigidity has anything to do with the latent period. It is doubtless due to a condition of the muscle that modifies the rate of tension development in its early stages.

*The latency of thickening responses.* After the readings of the records of the latency of muscle thickening (exclusive always of the period of rigidity) had been carefully made, and the results converted into absolute time units, the factor for delay of signal was added in each case. Then from this sum the corresponding end-plate delay (this latter at the corresponding temperature as indicated by a smoothed curve of the plotted results of Samojloff) was subtracted from the appropriate reading. The remainders of the several readings are plotted in the diagram (fig. 1B). None of these has a negative value. They approach $1\sigma$ only at the highest temperature, 30°C. They appear to fall about a smooth curve as shown in the diagram. Along with the "true" latencies are plotted also the mean values of Samojloff's end-plate delays as can be read off from a smoothed curve that I have constructed of his results. It will be noted that the two curves have quite different slopes. Thus we find that there still is left at all temperatures from 3.5°C. to 32°C., a considerable remainder of time that can only be called the true latency of the mechanical response. Temperature coefficients of the reciprocals of the mean values from the two curves at intervals of 10°, indicate that the underlying processes of the two phenomena are most probably quite different. (Compare the data in fig. 1, B and table 1.) This difference in rate of change reminds one of Kleinknecht's observations (1924), where it was shown that the influence of temperature on the latency of the onset of the mechanical effect is markedly greater than it is on the latency of the action potential.

*Experiments on muscle shortening.* In the second group of experiments where latencies of shortening were recorded, as stated above, a torsion-wire myograph was used. The mechanical-signal lever was dispensed with here and instead a thread-galvanometer was used as stimulus signal. The

tightened thread, with appropriate shunting and protecting resistances, was put in parallel with the muscle in the secondary coil of the inductorium. (Roos had his thread-signal in the primary coil.) Obviously no correction for signal lag need be made in these records. The number of experiments and successful photo-records was smaller than in the series on thickening of muscle and analysis of the material shows a variation among muscles (see table 2).

*Latency of action potential.* The Einthoven school has maintained that whatever the latency of the mechanomyogram may be it should be in no case more than the latency of the electromyogram. It is to be regretted that Roos did not include action potentials of the muscles along with his mechanograms.

TABLE 1

| TEMPERATURE | TIMES OF MOTOR END-PLATE TRANSFER (SAMOJLOFF'S)* | | REMAINDER DELAY IN THE MECHANOGRAMS OF MUSCLE THICKENING | |
|---|---|---|---|---|
| | $\sigma$ | $Q_{10}$ | $\sigma$ | $Q_{10}$ |
| °C. | | | | |
| 0 | 13.00 | — | — | — |
| 3 | — | — | 43.00 | — |
| 5 | 7.10 | 2.72 | 25.00 | 6.40 |
| 10 | 4.78 | 2.16 | 11.70 | 3.88 |
| 15 | 3.28 | 2.14 | 6.44 | 3.33 |
| 20 | 2.23 | 2.13 | 3.51 | 3.22 |
| 25 | [1.54] | [2.12] | 2.00 | 2.79 |
| 30 | [1.05] | [2.09] | 1.26 | 2.41 |
| 32 | [0.92] | — | [1.08] | — |

* The bracketed figures are data gotten by extrapolation.

Nevertheless in the literature there are several observations on action potential delays following moment of stimulation, and these usually follow stimulus to the intact motor nerves of the muscle. But since we now know the rate of nerve conduction and end-plate delay, wherever nerve lengths and temperatures are given, one may (upon adding the estimated further distance to neural equator of muscle) make reliable estimates for these delays which then may be deducted from the observed latencies with a considerable degree of assurance that the result will be nearly the true latencies. Fulton (1926) treated his data in this manner.

Such data I find also available in the very complete protocols of the experiments I carried out in a study especially of the form of the action potential in the single twitch of gastrocnemius muscle (1913). It may be said at once that the "latencies" incidentally mentioned in that paper do not include the corrections just discussed and therefore are not to be considered as "true" latencies. In addition it should be stated that, in

printing, the photographic records there exhibited were reproduced so poorly that one gets no idea of the excellence of the original photographs. In that series of experiments a few records were taken of curarized muscles, with stimulating electrodes applied to the patellar third of the muscle, and non-polarizables (leading to the thread galvanometer) applied to the Achilles half of the muscles. In all these records, although the poles of the galvanometer were alternately reversed, the action potential moves always

TABLE 2

*Latency of shortening response of muscle*

| DATE OF EXPERIMENT | TEMPERATURE | DELAY BETWEEN PEAK OF STIMULATION SIGNAL AND MUSCLE FEVER RISE, IN $\sigma$ (a) | CORRESPONDING END-PLATE DELAY IN $\sigma$ (b) | TRUE LATENT PERIOD (a−b) $\sigma$ |
|---|---|---|---|---|
| | °C. | | | |
| *v/ 9/35* | 10.3 | 7.13 | 4.65 | 2.40 |
| | 11.5 | 6.40 | 4.30 | 2.10 |
| | 24.5 | 3.40 | 1.50 | 1.90 |
| | 28.0 | 2.25 | 1.00 | 1.25 |
| | 31.0 | 1.67 | 0.70 | 0.97 |
| *xi/ 2/35* | 8.5 | 8.90 | 5.35 | 3.55 |
| | 8.6 | 8.14 | 5.30 | 2.84 |
| | 17.6 | 3.82 | 2.72 | 1.10 |
| | 18.0 | 3.35 | 2.65 | 0.70 |
| | 12.0 | 6.41 | 4.15 | 2.26 |
| | 12.5 | 7.11 | 3.96 | 3.15 |
| *xi/26/35* | 25.0 | 3.91 | 1.4 | 2 5 |
| | 25.0 | 3.91 | 1.4 | 2 5 |
| | 15.3 | 8.51 | 3.3 | 5.2 |
| | 14.0 *ca* | 9.03 | 3.6 | 6.4 |
| | 25.0 | 3.90 | 1.4 | 2.5 |
| | 25.0 | 3.59 | 1.4 | 2.2 |
| | 25.0 | 3.92 | 1.4 | 2.5 |
| | 15.5 | 8.09 | 3.2 | 4.9 |
| | 15.3 | 7.76 | 3.3 | 4.5 |
| | 15.5 | 7.76 | 3.2 | 4.6 |
| | 8.5 | 16.07 | 5.3 | 10.8 |

promptly with the moment of stimulus, and no latencies of the onset of the electromyograms are observable. After deducting for delays of axon conduction according to Snyder (1931) and end-plate delays according to Samojloff (*loc. cit.*), it appears that the mean latency of onset of action potential (in the isometric twitch) in 30 observations, at about 20°C., is $0.7\sigma$; in 14 observations at 28°C., $0.5\sigma$; in the isotonic twitch, 16 observations at 28°C., the mean latency is but $0.2\sigma$. These remainders are too

small perhaps to attach much significance to their absolute values. But the findings of recent workers corroborate them, and the fact that the values are affected by temperature is positive evidence that there is a "true" latency of electric response, however small it may be. The longer latencies of action current observed by Kleinknecht doubtless are due either to the fact he curarized his muscles, or to delay of end-plates that were not paralyzed, none of which delays apparently had been deducted.

The duration to peak of the first deflection of the diphasic action potential shows no real difference between the isometric and isotonic twitches. Of 19 observations at 28°C. the mean duration is $4.2\sigma$; of 28 observations at 20°C. the mean value is $5.4\,\sigma$.

Discussion. It will be noted that these times of the electric response also agree with earlier (Burdon-Sanderson) and later observations (Fulton, 1926, p. 60). If excess time to first peak of what we call action potential of muscle should be added to the motor-end-plate delay and therefore also subtracted from the above gross latency readings (as we would be forced to do if we accept Lindhard's position) then there would be a negative remainder, and we are forced either to the *reductio ad absurdum* that muscle begins to contract before it is excited, or to the doctrine that the action potential does not really precede or accompany but rather follows a state of excitation. This latter view is the logical conclusion also if we accept the view of Lindhard (1932) and part of the teaching of the Einthoven School. If there is no real electrical response of contractile tissue (Lindhard) and if the contractile response begins simultaneously with the onset of the observed electrical response (Einthoven School) then the electric response must be a delayed expression of an event that actually preceded the onset of the contractile act. Obviously we cannot accept either of these conclusions.

We finally have to consider the argument that there are probably internal factors (*e.g.*, elastic stretch and viscosity) which delay the external expression of change of form on the part of the muscle organ, and that these alone may account for the observed latency of the muscle twitch. It is known that the elasticity modulus of skeletal muscle increases with decrease of temperature (compare Wöhlisch, 1928–31). If this factor has any effect at all it ought therefore to decrease the latent period with decrease of temperature. On the other hand the viscosity of muscle fluids doubtless is increased with decrease of temperature, probably only to a small extent since the viscosity of other body fluids have $Q_{10}$ values of the order of 1.3 (compare Snyder and Todd, 1911). It has been argued that since some substances (quite foreign to muscle), such as ricinus oil, have viscosities whose values of $Q_{10} = 2.5$, muscle fluids also are probably affected by temperature to a similar degree. But until this is proven to be the case we shall have to reject it, and assume the more probable case that the $Q_{10}$

of the viscosity of muscle fluids is of the order of other body fluids. While there can be no doubt that viscosity plays an important rôle in the time factor of contraction and relaxation, that is, in form changes of muscle, that should not lead us to infer that viscosity also plays a major rôle in the pre-contraction period. The evidence adduced that viscosity plays a much greater rôle during the period of relaxation than during the period of tension development (Bolzer, 1933) would lead us to suspect that during the latent period, a period still more antecedent, viscosity would play still less a rôle. What we know from direct measurements of viscosity on protoplasma and colloidal systems also does not warrant our laying much stress on the rôle of viscosity during the latent period. Heilbrun (1924, 1928) showed that the viscosity coefficient of protoplasm in Amoeba and Cumingia eggs actually remains constant over certain ranges of temperature and actually rises with rise of temperature in certain other ranges. Stiles (1930) from his review of the influence of viscosity on rates of diffusion of substances in solution, including colloid systems, finds "actually the coefficient of diffusion is reduced relatively little for considerable increases in the viscosity of the system," and concludes that "viscosity of protoplasm so far from determining the rate of biological reactions generally plays a very small part, perhaps a negligible part, in determining such reactions."

It is now almost certain that however much, in the evolution of metozoa, development of nerves has reduced the time interval between the moments of stimulus and response, there still remains a short path of humoral transmission, so far as the autonomic system is concerned. While there is no evidence of the liberation of a humoral transmittor due to direct stimulation of somatic muscle, yet since the essential quality of the nervous impulse, so far as we know it, is that of a wave of potential difference, we are justified for the present in entertaining the view that our electrical stimulus acts on the muscle in the same way.

That excitation itself induces initially chemical changes, however is made probable by the work of Furnhjelm, Hortling and Renquist (1934), who show that under given conditions the dependence of duration of latent period on stimulating strength and tension development may be expressed by a formula representing bimolecular chemical reactions, a formula that Renquist had previously shown to be similar to Hoorweg's equation expressing the relation between stimulation and the rheobase parameter of muscle.

Finally the question as to whether the requisite chemical changes could possibly take place in intervals as brief as those observed in muscle latencies is answered by Barcroft (1935) in his address on "The velocities of some physiological processes." In this he cites the work of Millikan on myoglobin and cytochrome where $\frac{1}{2}$ oxidations are observed to occur in

0.001 second and less, and the work of Warburg and Kubawitz on Atmungsferment, from which it appears that one molecule of $O_2$ can be dealt with in $1.10^{-5}$ to $1.10^{-6}$ second at temperatures from 10° to 40°C. There can be no doubt that other kinds of chemical changes can proceed in such brief intervals of time, and that the briefest periods observed for muscle latencies are quite ample for the hypothetical chemical changes.

## SUMMARY AND CONCLUSION

The problem of the latency of skeletal muscle has been reinvestigated on frog's gastrocnemius muscle at different temperatures.

1. A method was adopted which involves increasing the speed of the photographic recording film along with the increase in the rate of the muscle's contraction as effected by increase of temperature. This tends to keep the angle, made by the rising muscle lever with its base line, constant. In case the number of readings at each temperature is not always the same, the errors in the readings thus also tend to be as often negative as positive and therefore cancel each other. Improved muscle levers were built and made quite as sensitive it is believed as any heretofore described. Temperature controlled muscle-chambers were made small and the essential parts of the levers and the recording apparatus could not be subjected to temperature changes.

2. The readings of the latencies from the records were corrected for all delays not clearly belonging to the muscle fibers, such as the delay of the stimulus signal and motor end-plate delay. But even so, and in spite of the care in method and technique there is still left a positive remainder, a "true" latency, which varies greatly and inversely with temperature, as has been observed heretofore by so many other investigators.

3. From measurements made on an older series of latent period records of action potentials of muscles stimulated through their nerve (and after deductions made from axon conduction and motor end-plate transfers) it is found that a, there is a small positive remainder which may be interpreted as a "true" latency of electric response, and b, since the true latencies of the mechanograms of the present experiments are so much longer it is concluded there is ample time for the action potential to reach the peak of its first deflection at all normal temperatures below, say 20°C., before the mechanical response begins.

4. It is concluded that the evidence from these experiments favors the view that the onset of electric response of the frog's gastrocnemius muscle precedes the onset of the mechanical response.

5. A brief discussion of some of the changes that have been suggested to take place during the observed true latency of muscle follows. It is concluded that they are probably largely chemical rather than purely physical in their nature and that the latency is a property of the muscle

fibers themselves rather than interfibral tissue, and thus a real part of the contractile response.

## REFERENCES


BARCROFT, J. In reports of Internat. Physiol. Congress, Moscow, 1935.

BOZLER, E. Protoplasma **19:** 293, 1934.

EINTHOVEN ET HUGENHOLTZ. Arch. Neerlandaise **5:** 174, 1901.

FULTON, J. F. The contraction of muscle. Baltimore, p. 84, ff, 1926.

FURNHJELM, HARTLING AND RENQUIST. Skand. Arch. **70:** 88, 1934.

VAN DER HEYDE, H. C. Thèses . . . a la Faculté de Sciences de l'Université de Paris. Part B. Paris. p. 103, 1923.

DE JONGH, C. L. Pflüger's Arch. **213:** 216, 1926.

KLEINKNECHT, F. Ztschr. f. Biol. **81:** 25, 1924.

LINDHARD, J. Skand. Arch. f. Physiol. **64:** 299, 1932.

ROOS, J. Pflüger's Arch. **229:** 15, 1932; J. Physiol. **74:** 17, 1932.

SAMOJLOFF, A. Pflüger's Arch. **208:** 508, 1925.

SNYDER, C. D. Ztschr. f. Allg. Physiol. **14:** 265, fig. 1, 1912.
This Journal **32:** 336, 1913.
Quart. Rev. Biol. **6:** 289, 1931.

SNYDER AND TODD. This Journal **28:** 167, 1911.

WERTHEIM-SALOMONSON, I. K. A. Kon. Akad. van Wetensch. te Amsterdam, Proc. **21:** 1152, 1919.

WÖHLISCH, E. Ztschr. f. Biol. **88:** 52, 1928.

WÖHLISCH, E. AND H. G. CLAMANN. Ibid. **91:** 431, 1931.

ZELDENDRUST, S. Onderzoek. physiol. Lab. Reiks Univ., Utrecht, 7 (Reihe 2) p. 245, 1933.

# RELATION OF THE URINE CHLORIDE RATE TO THE PLASMA CHLORIDE CONCENTRATION BEFORE AND AFTER ADMINISTRATION OF SODIUM CHLORIDE

EATON M. MacKAY AND LOIS LOCKARD MacKAY

*From The Scripps Metabolic Clinic, La Jolla, California and The Department of Medicine, Stanford University Medical School, San Francisco*

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The study described in this paper is the beginning of an attempt to determine some of the factors which govern the rate of excretion of inorganic elements by the kidney. The experiments were designed to determine the relation of the rate of chloride excretion in the urine to the plasma chloride concentration.

In an attempt to induce constancy of the factors other than the plasma chloride concentration which might affect the rate of chloride excretion as in the case of urea (Addis and Drury, 1923), a diuresis was induced by the administration of water and urea. Water alone was satisfactory unless NaCl was administered when fluid retention resulted and so urea was used in both groups. In regard to the production of a diuresis in the rabbit recent investigators (Kaplan and Smith, 1935) report that in this species the excretory ratios of all the substances they studied, even urea, increase with the rate of urine flow and fail to reach a constant value at urine flows which apparently are maximal. Furthermore these workers found that water intoxication occurs easily and frequently in the rabbit when large quantities of water are administered in an effort to obtain high urine volumes. We simply wish to point out that their conclusions do not always apply and seldom hold when water alone, urea and water and urea, small quantities of creatinine and water are administered to rabbits per os and the excretory ratios of urea or urea and creatinine (after creatinine is administered) measured. Concerning water intoxication in the rabbit and hydremia and anuria in the dog we can support them. We have frequently observed a reduction in urine flow, even anuria and at times death from "water intoxication" following the intravenous administration of a considerable quantity of either creatinine or inulin particularly when urea, which is of great assistance in maintaining a diuresis, had not been given. Kaplan and Smith (1935) indicate that intravenous injections favored water intoxication in rabbits and suggest that excessive dilution of the plasma is responsible. This seems very likely for Van Slyke, Hiller and

Miller (1935) have reported that inulin does not enter the erythrocytes and that creatinine does so only slowly. It is possible that a similar lack of diffusibility applies to other cells and such a property would be bound to cause a marked hydremia of the plasma.

METHODS. Healthy male rabbits were chosen for these experiments. The procedure was identical for every animal in a group. No food was given for fifteen hours before the experiment was commenced. Three hours before the first catheterization 30 cc. per kilo body weight of a 5 per cent solution of urea were given by stomach tube, and every hour thereafter until the experiment was ended 30 cc. of water per kilo were administered in the same manner. Four to six hourly observations were made in each experiment. In the group receiving salt 2.5 grams of NaCl per kilo were given with the water an hour after the urea.

The urine was obtained by catheter at intervals of approximately one hour. After each catheterization the bladder was thoroughly washed with distilled water. Chloride and urea were determined separately on the specimen and the washings in each case so that the urine volume could be accurately known.

Arterial blood was obtained from the left heart at the middle of each period of urine collection and a part was centrifuged immediately under paraffine oil. A small amount of heparin was used as an anti-coagulant on the assumption that it would have less effect on the distribution of chlorides between the cells and the plasma than a calcium precipitant. Chlorides were determined on the plasma by Van Slyke's method (1924). Whitehorn's method (1921) was used for the determination of urine chloride. In some experiments urea was determined in whole blood and urine according to the methods described by Addis (1925). The results are expressed as milligrams NaCl per kilo body weight per hour and as milligrams NaCl per 100 cc. of plasma.

RESULTS. In figure 1 have been plotted the results on the first group which were given no extra sodium chloride. In the upper portion of this figure have been plotted the urine urea excretion and blood urea concentrations determined simultaneously with the chloride data plotted below. The straight line through the observations represents the average relationship between the urea figures. The urea chart because it represents an enlarged area of what should be a larger graph is misleading in that the variability appears greater than it actually is. Since the question of reaching a constant figure for urea in the rabbit has been raised (Kaplan and Smith, 1935) it might be noted that the maximum variability for any one animal was 14 per cent over the 4 to 6 hours observations were made. It should be borne in mind that these measurements were made during a good diuresis (see lower portion of fig. 1) and after the administration of a rather large dose of urea.

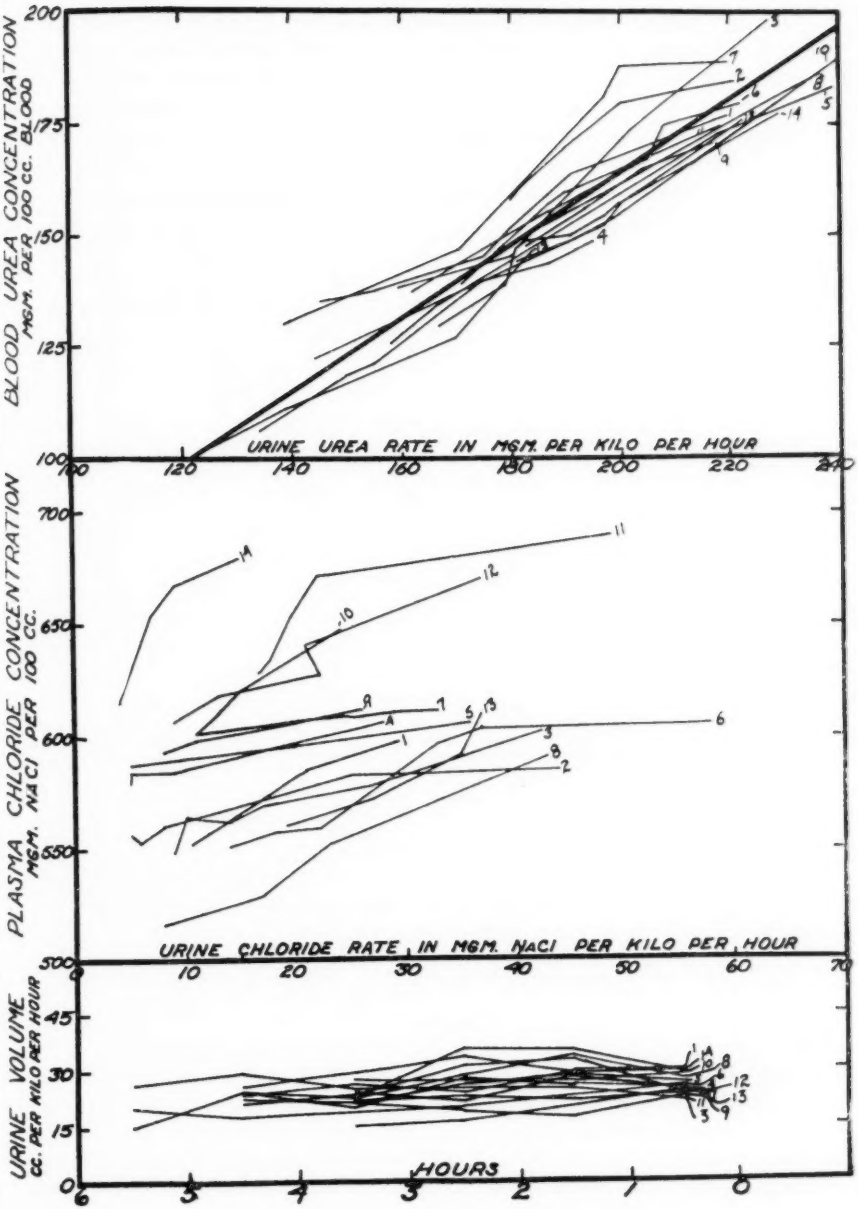
BLOOD UREA CONCENTRATION MGM. PER 100 cc. BLOOD
200
175
150
125
100
URINE UREA RATE IN MGM. PER KILO PER HOUR
100
120
140
160
180
200
220
PLASMA CHLORIDE CONCENTRATION MGM. NaCl PER 100 cc.
700
650
600
550
500
URINE CHLORIDE RATE IN MGM. NaCl PER KILO PER HOUR
10
20
30
40
50
60
URINE VOLUME cc. PER KILO PER HOUR
45
30
15
0
HOURS
6
5
4
3
2
1
0

Fig. 1

In the middle of figure 1 are the data on chloride excretion. All that can be gathered from this graph is that there is a general tendency for the higher rates of chloride excretion to be associated with the higher concentrations of chloride in the plasma. It is obvious that there is a basic difference between the excretion of chloride and urea. The chief dif-

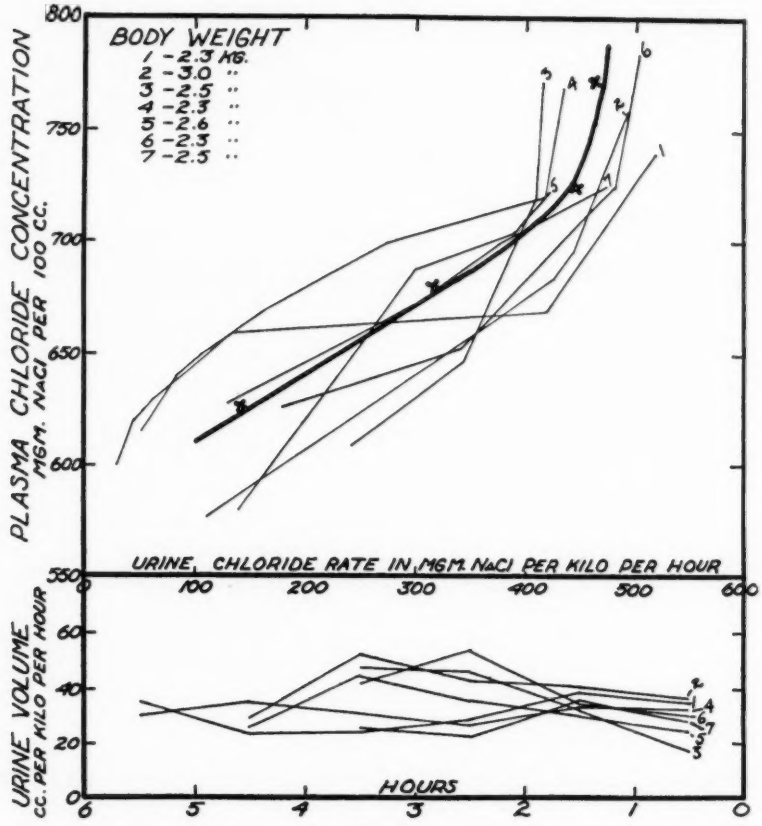


Fig. 2

ference, namely, that some of the chloride which is filtered through the glomeruli is reabsorbed by the renal tubules, has been demonstrated by Wearn and Richards (1924), Oliver and Shevky (1929) and others. If urea is reabsorbed by the renal tubules the reabsorption rate must be proportional to the rate of urea excretion for the latter under the conditions of the experiments is directly proportional to the blood urea concentration.

The rate of urea excretion then is determined almost entirely by the rate of glomerular filtration (Van Slyke, Rhoads, Hiller and Alving, 1934). Since this is practically constant it is reasonable to believe that the glomerular rate of chloride excretion is in all probability directly proportional to the plasma chloride concentration. The inconstancy of the relation of the bladder rate of chloride excretion to the plasma chloride concentration is then due to variation in the rate of chloride reabsorption by the tubules. Believing that part of this variation might be due to differences in the amounts of the different cations excreted with the chloride and which have variable reabsorption characteristics observations were made following the administration of large doses of sodium chloride. These form figure 2 and show a greater tendency to uniformity than when no salt was given. Neither the individual sets of observation nor the average figures indicate a constant relation of the urine chloride rate to the plasma concentration. The nature of the average relationship is in general curvilinear and may be significant but should be considered together with data which are not now available.

Our results suggest that the most satisfactory study of chloride excretion would be along with certain cations particularly sodium and potassium. Further than this it is probable that a real intelligible picture will be available only when the excretion of these ions is observed in connection with glomerular filtrate volume studies assuming that this figure may be obtained from inulin (Van Slyke, Hiller, and Miller, 1935). In other words a study of the factors which govern the rate of tubular reabsorption of chloride, as measured indirectly, might prove more fruitful than experiments involving only the chloride of the bladder urine. Certain calculations in this regard might possibly be made from our urea data if it were not for the fact (Kaplan and Smith, 1935) that the relation between the urea and inulin ratios is inconstant or, at present at least, uncertain.

*Chloride threshold.* Aitkin (1929) has reported experiments on man which lead him to conclude that there is no threshold for chloride excretion. That for the rabbit at least there is a threshold for chloride excretion in the sense that the rate of reabsorption of chloride by the renal tubules may become equal to the rate of excretion of chloride by the glomerular filtrate so that the bladder urine becomes free of chloride, there can be no question. Four rabbits which had undergone chloride depletion by a 9 to 12 hour diuresis as in the experiments described here were kept overnight without food and again subjected to the same treatment. After 4 to 6 hours of a repetition of the urea diuresis with urine volumes of 25 to 30 cc. per kilo per hour, the urines of all four rabbits became chloride free so that there was not even the faintest opalescence when they were tested with a nitric acid-silver nitrate solution. The chloride free urines were obtained at plasma chloride concentrations of 490, 493, 494, and 500 mgm. NaCl per

100 cc. respectively. It is interesting that in some of the experiments of the preceding day plasma chloride concentrations considerably below these figures were obtained without any sign of the bladder urine becoming chloride free. We interpret this as the possible result of the excretion of notable amounts of potassium chloride on the first day which by the time the second series of observations were made had almost entirely been washed out of the organism so that we were dealing with sodium chloride excretion when the threshold was obtained.

## SUMMARY

Under certain conditions with a high rate of urine flow when there is a direct relation of the rate of urea excretion to the blood urea concentration there is no semblance of uniformity in the relation of the urine chloride rate to the plasma chloride concentration. This is interpreted as being due to fluctuations in the rate of chloride reabsorption by the renal tubules.

Following the administration of large doses of sodium chloride with higher plasma chloride concentrations there is a greater tendency for the rate of excretion of chloride in the urine to follow the plasma chloride concentration. The relationship appears to be curvilinear.

The rabbit definitely has a chloride threshold. At high urine volumes chloride free specimens were obtained at an average plasma NaCl concentration of 494 (490–500) mgm. per 100 cc.

## REFERENCES

ADDIS, T. AND D. R. DRURY. J. Biol. Chem. **55:** 105, 1923.
J. Lab. and Clin. Med. **10:** 402, 1925.
KAPLAN, B. I. AND H. W. SMITH. This Journal **113:** 354, 1935.
OLIVER, J. AND E. SHEVKY. J. Exper. Med. **50:** 15, 1929.
J. Exper. Med. **50:** 601, 1929.
VAN SLYKE, D. D., A. HILLER AND B. F. MILLER. This Journal **113:** 629, 1935a.
VAN SLYKE, D. D. J. Biol. Chem. **58:** 523, 1924.
VAN SLYKE, D. D., C. P. RHOADS, A. HILLER AND A. ALVING. This Journal **110:** 387, 1934.
VAN SLYKE, D. D., A. HILLER AND B. F. MILLER. This Journal **113:** 611, 1935b.
WEARN, J. T. AND A. N. RICHARDS. This Journal **71:** 209, 1924.
WHITEHORN, J. C. J. Biol. Chem. **45:** 449, 1921.

# A STUDY OF MOTOR COÖRDINATION AND TONUS IN DEAFFERENTED LIMBS OF AMPHIBIA

PAUL WEISS[1]

*From the Department of Zoology, The University of Chicago*

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It is generally agreed that in man and mammals the motor functions are seriously impaired when the proprioceptive control of movements has been eliminated by disease, accident or operation. Movements requiring precise adjustments, such as oral and facial movements, suffer most (Bell, 1821; Schöps, 1824; Filehne, 1886; Exner, 1890; Pineles, 1891). In the hind limbs (dog, monkey), the elimination of sensory control by transection of the dorsal nerve roots (Baldi, 1885; Mott and Sherrington, 1895; H. E. Hering, 1896; Bickel, 1897) at first abolishes the coördinated use of the affected limbs, although later functional capacity may be restored with the visual sense acting vicariously for the lost proprioceptive sense.

We see that in order to secure a coördinated movement, it is not necessary that the sensory control be exerted by the very same muscles and joints that perform the movement. This point is further illustrated by the results of the deafferentation of the fore limbs. A monkey is reluctant to make use of his deafferented arm, but if he can be induced to do so, the movements, although lacking the normal precision, are essentially coördinated (H. E. Hering, 1897; Munk, 1903). A pigeon with one wing deafferented shows almost no symptoms distinguishing it from a normal pigeon in flight and postural reactions, although bilateral deafferentation does abolish the ability to fly (Trendelenburg, 1906). There are even organs the motor functions of which are left completely undisturbed by the removal of their sensory control, such as, e.g., the tail of the dog, which after transection of all its dorsal roots is still carried and moved in the normal way (Merzbacher, 1902a).

The fact that motility in mammals is impaired in various degrees when the centers are prevented from receiving sensory messages, was recognized by Cl. Bernard (1850), and has been elaborated by Exner (1894) into a theory of "sensomotility." But the experiments indicate clearly that, as far as the organization of the motor patterns is concerned, the rôle of sensory control is not a fundamental one. The gross and fundamental patterns

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for a limb survive the complete elimination of its own sensory nerve supply, and the movements of such limb remain aimed and coördinated. What is found to be affected by the loss of the sensory control is not the existence of a given motor pattern as such, but only its precision and finer adaptability; moreover, there is a tendency to omit the use of the ill-controlled limb in the integrated activity of the animal.

However, in some of the literature touching upon the subject one cannot fail to observe a certain overemphasis on the importance of proprioceptive phenomena in higher vertebrates, in spite of the experiments mentioned, and although the intracentral origin of motor patterns has been repeatedly asserted (Gr. Brown, 1914, and others). In line with this trend, the proprioceptive component of motility has recently been emphasized also in Amphibia. On several occasions, the belief was expressed that the so-called phenomenon of "homologous function" in transplanted amphibian limbs, discovered by the author and interpreted as evidence for a "resonance" principle of nervous communication (for literature see P. Weiss, 1935), might perhaps be explained by assuming that, under the guidance of proprioceptive excitations from the transplanted limbs, the centers may have been induced to develop such new patterns as result in the observed peculiar type of function. In other words, proprioceptive effects were again claimed to be instrumental in the laying down and shaping of motor patterns. This assumption, while originally mere conjecture, seemed to receive support from a number of interesting experiments reported in two recent papers, one by Detwiler and Vandyke (1934) and the other by Nicholas and Barron (1935).

Detwiler and Vandyke undertook to determine whether or not sensory control is essential in the first establishment of motor function in the embryo. Working on salamander embryos (Amblystoma), they removed the dorsal part of the developing neural tube which, according to Harrison (1924), contains the material concerned with the formation of the spinal ganglia. In limbs thus innervated by purely motor nerves, motility was lacking. They were carried in the rigid posture of extreme adduction and extension without taking part in locomotion. The failure to develop motor functions other than this tonic rigidity was ascribed by the authors to the absence of sensory innervation, and it was concluded that for the development of coördinated function in a limb the integrity of its sensory control is indispensable.

Nicholas and Barron arrived at a similar conclusion after experiments of a different type. According to the authors, the presence of the afferent nerve supply from a developing limb is necessary in order to set up reflex connections with the contralateral limb. Furthermore, by stimulation experiments on normal salamanders, the authors believe they have demonstrated that every dorsal root has reflex connections exclusively with the

motor nuclei of its own segment and not even with the adjacent segments contributing to the same limb plexus. All of which, according to these authors, points to the influence of proprioceptive innervation on both the establishment and the functioning of motor coördination.

All previous experiments on amphibia had suggested that, in the basic motor functions of this group, the rôle of sensory control is even less important than it was thought to be in mammals. Slight disturbances in the coördination of frogs with deafferented hind legs, as described by the earliest investigators in the field (Panizza, 1837; Stilling, 1842), were obviously due to inadequate operative methods entailing injury to the spinal cord. All subsequent experimentation (Brown-Séquard, 1850; H. E. Hering, 1896; Bickel, 1897) proved that coördination of movements in the frog is not essentially impaired by transection of the sensory nerve roots.

The views of Detwiler and Vandyke and of Nicholas and Barron, attributing a dominant rôle to proprioceptive control, do not find support in these earlier observations. For reasons which will be discussed later, the experiments of Nicholas and Barron cannot be regarded as conclusive. However, the results of Detwiler and Vandyke are absolutely convincing, and yet they appear to conflict with the results obtained on deafferented frogs.

Out of this unsettled state of affairs arose the necessity for a reinvestigation of the whole problem. This was undertaken in the present study for which the toad and salamander were used. These two forms were chosen because they have chiefly served in the study of the phenomenon of "homologous function." It was intended 1, to investigate the effects of deafferentation on the toad and the salamander, and 2, to examine whether or not the appearance of "homologous function" in transplanted limbs depended on the presence of sensory innervation. Only the first point is dealt with in this paper.

*A. Experiments on the toad.* Specimens of Bufo americana and Bufo fowleri of from 5 to 7 cm. body length were used. The animals had been kept in captivity for a few weeks before the operation but were all in excellent condition. Ether was used for anesthesia. The deafferentation was done by a method which did not require the opening of the vertebral canal. Access to the spinal ganglia and the dorsal roots was gained from the outside of the intervertebral foramina, as described in an earlier paper by the author (1935). The exposed spinal ganglion after being severed from its peripheral sensory nerve and from the adjacent motor root, can easily be pulled out trailing its dorsal root behind it. If properly done, the operation does not cause extensive bleeding. The dorsal roots are so loosely connected with the spinal cord that their pulling out has no injurious effects (P. Weiss, 1934, 1935). Deafferentation, as described here, consists of complete extirpation of the spinal ganglia and their attached dorsal roots.

The hind legs of the toad are innervated from the eighth, ninth and tenth segments. In order to exclude errors which might result from the contribution of aberrant branches to the plexus from neighboring segments, the seventh and eleventh[2] nerves were cut at their roots in addition to the deafferentation of the eighth, ninth and tenth nerves. The fore limb plexus is formed by the second, third and fourth nerves. Here deafferentation was performed only on the third and fourth nerves, while the second nerve, which adds but a minor branch to the plexus, was cut completely. In a number of hind limb deafferentations, the sympathetic innervation was also removed by excising the sympathetic chain including the seventh, eighth, ninth and tenth ganglia, and the corresponding rami. The deafferentation completed, the separated os ilium and sacral diapophysis were reunited by means of a stitch of silk, in order to prevent sagging of the legs; finally the skin was sutured.

The complete absence of sensitivity to any sort of stimulation in the limbs served as proof that the operations had been radical and successful. The animals were subjected to the first functional examinations immediately after the effects of the anesthesia had subsided, i.e., a few minutes after the resuming of breathing movements and the appearance of the lid reflexes. From then on they were kept under daily observation until 22 days after the operation. The functional examinations, unless otherwise stated, were done on animals with intact brain.

*1. Deafferentation of hind limbs* (31[3] cases, including 11 with unilateral and 20 with bilateral deafferentation). *Resting posture.* Animals with deafferented hind limbs assume the typical resting posture with the hind legs extremely adducted and flexed. At first glance no difference from normal can be discerned. Closer inspection reveals a difference in the posture of the foot which often fails to be pressed against the ground as closely as in normal animals. This difference has already been described for the frog by H. E. Hering (1897) and by Merzbacher (1902).

*Locomotion.* All three types of locomotion—jumping, walking and swimming—are executed in perfect coördination after unilateral or bilateral deafferentation. The jumps are vigorous and aimed. In unilaterally deafferented specimens no deviation to the operated side was ever noticed. Yet in bilaterally deafferented specimens it sometimes appeared as if the distances of the individual jumps were slightly subnormal, this being the only impairment observed in jumping on rough ground. On smooth ground, the inferiority of deafferented limbs becomes more marked, inasmuch as the feet frequently slip, owing to the lack of close contact between

[2] The eleventh nerve is not always present.

[3] The numbers of cases reported in this paper are larger than those in the preliminary communication (Proc. Soc. Exper. Biol. and Med. **32:** 436, 1935), as some additional experiments have been performed since that note was published.

their plantar surfaces and the ground at the onset of the jump. If a jump happens to end in a slightly abnormal posture of the foot, proprioceptive perception being abolished, no postural correction follows until the next active move brings it back to normal. Slow locomotion on land was occasionally achieved by walking in steps, the two hind legs alternating. Again in this type of locomotion no striking difference from normal could be noticed after bilateral, nor any asymmetry after unilateral deafferentation. The same holds true for the swimming movements which were begun as soon as the animals were immersed in water.

*Reflexes.* Righting, turning and tactile reflexes were studied. Toads turned to their backs return to the normal posture chiefly by hind limb activity, in one quick movement over one side. Normal animals, specially tested for that purpose, showed no preference for either side. In animals with deafferented hind limbs, the righting from the back was performed as quickly and—on rough ground—as adroitly as in normal specimens. In unilaterally deafferented specimens the righting is effected as frequently with the deafferented as with the normal limb.

A toad, subjected to slow horizontal rotation on a turn table, tends to compensate the rotation by extending the legs of one side and flexing those of the opposite side. This turning reflex is exhibited by deafferented legs as well as by normal legs, in unilaterally deafferented specimens equally well on both sides.

Local chemical irritation of the skin of the trunk by ammonia or acid evokes an aimed wiping reflex of the deafferented limb of the corresponding side, such as has already been seen in the frog by Bickel (1897).

One reflex, however, failed to appear after the deafferentation of both hind limbs, namely, the burrowing reflex. Normal toads have the habit of digging into soft soil with their hind legs, even under laboratory conditions. Apparently the factor eliciting the reflex is a perception of the soft consistency of the ground, mediated through the receptors of the hind limbs. Since in unilaterally deafferented animals the perception is still possible by way of the intact limb, the reflex appears in these animals in the usual fashion, the deafferented limb taking an equal share in the digging. Only the elimination of sensation in both limbs, as in bilaterally deafferented specimens, abolishes the reflex. It is obvious, therefore, that what has been destroyed by bilateral deafferentation is not the pattern of coördinated movements, but merely the source from which it can be called forth.

The sympathetic chain was removed in 5 of the 11 unilateral deafferentations, and among the bilateral deafferentations on both sides in four cases, and in three cases on one side only. The loss of the sympathetic innervation has no effect on the motor behavior, as far as can be detected from gross observation.

*Tonus.*[4] Since the well-known experiments of Brondgeest (1860) on frogs with deafferented hind limbs, an important rôle in the maintenance of postural tonus has been ascribed to the sensory excitations coming from the limbs. The toad, in which tonic reactions are much more conspicuous than in the frog, offers itself as a particularly favorable object for the study of this type of tonus.

Not all toads are equally well suited for the study of tonic reactions. There are two types of individuals which differ considerably in this respect. They may be called the motile and the tonic type. The motile type reacts to stimuli predominantly with locomotion or struggle, more or less in the manner of a frog; whereas the tonic type tends to fall into a sort of cataleptic rigor characterized by extreme flexion of head and legs. One method of telling the two types apart is to turn the animals on their backs: A motile animal rights without delay, while a tonic animal merely flexes its legs and remains on its back for several seconds or even minutes before righting. Correlated with its righting habit is the reaction of an animal when vertically suspended in free air. While the motile animal drops the hind legs after an initial flexion to a half-extended position, the tonic animal keeps them retracted in the flexed position for periods lasting as long as several minutes. Only animals of the tonic type were used to examine the effects of deafferentation on tonus.

The tests were as follows: Adhesive tape was wrapped around head and fore legs, leaving free the parts posterior to the shoulder level. By means of a strap fastened to the tape the animal was then suspended vertically from a hook, head up. By slight pressure on the body the animal can be brought to retract its legs, in which position they are kept by a normal tonic animal for some time. Eventually, the limbs drop in gradual relaxation or perform a wiping or struggling movement which may end either in relaxation or in renewed retraction. Prior to relaxation, however, the flexor tonus has been sufficient to hold the legs retracted against their own weight for some length of time.

Contrary to anticipation, it was found that this tonic capacity was fully retained after deafferentation. In repeated examinations, suspended animals were observed to hold their deafferented hind limbs retracted to the body for periods lasting sometimes several minutes (fig. 1). Within this whole period the animal is perfectly quiet. The end of the period is marked either by a spontaneous movement or by a gradual drooping of the legs into a half or wholly relaxed position. The drooping occurs first in the wrist, often as early as a few seconds after the retraction, while the other

[4] The vagueness and the indiscriminate use of the term "tonus" have often been criticized, most recently very candidly by Cobb and Wolff (1933). In the present paper the term will be employed simply for convenience, to designate a maintained muscular contraction.

joints follow much later. As a rule, both legs relax simultaneously. The longest recorded maintained flexion in deafferented limbs lasted for nine minutes. In some animals, the tonus of the deafferented legs was sufficient to carry, in addition to the weight of the legs, a light wire clamp attached to the foot. The other extreme was found in animals where only the proximal muscles developed enough tonus to hold up the leg, while the muscles of the ankle failed to maintain a prolonged dorsal flexion of the foot. This kind leads over to the non-tonic animals which can hold neither leg nor foot.

Fig. 1

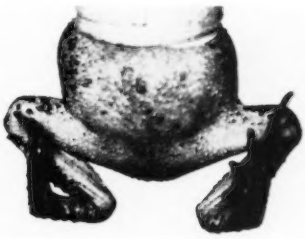

Fig. 2

Fig. 1. Photograph of case S17, taken 16 days after the bilateral removal of the spinal ganglia and dorsal roots 8, 9 and 10; nerve pairs 7 and 11 severed. Maintained tonic flexion of the deafferented hind limbs in the vertically suspended animal. The photograph was taken 60 seconds after the flexion of the limbs, within which time the animal did not move.

Fig. 2. Ventral view of case S26, taken 5 hours after the removal of the left spinal ganglia and dorsal roots 8, 9 and 10 and left sympathectomy. Symmetrical tonic flexion of the deafferented hind limb (right in the picture) and the intact limb (left in the picture) maintained for 30 seconds in the suspended animal.

The observations made on bilaterally deafferented hind limbs are fully borne out by the results of unilateral deafferentation. In truly tonic animals, no trace of asymmetry in the posture of the retracted deafferented leg and the control leg of the opposite side has been observed (fig. 2). Both legs, the normal and the deafferented, were kept retracted for the same length of time, both finally relaxing simultaneously, although in some cases the drooping was slightly more extensive in the deafferented leg.

One must conclude from these observations that in the hind limbs of the toad (tonic type) no appreciable loss of tonus is caused by deafferentation. Proprioceptive reflexes, even though under normal conditions they may contribute to the maintenance of the tonus, are certainly not essential for it.

The same holds in regard to the sympathetic innervation. It had been claimed (de Boer, 1913), that tonic innervation in the frog is mediated

through sympathetic fibers. This view has met with considerable opposition since the experimental evidence is predominantly against it (compare Bremer, 1932). It certainly cannot draw any support from our experiments. Nine animals in which bilateral or unilateral deafferentation had been combined with the destruction of the corresponding sympathetic ganglia and their connections proved to be just as capable of prolonged retraction of their legs as after simple deafferentation (fig. 2). In three animals with bilaterally deafferented hind limbs the sympathetic innervation was removed on one side, the other side serving as control. But no consistent difference could be noticed in the suspension tests. The duration of the tonic retraction was the same for both limbs, lasting in one specimen up to three and a half minutes, in another specimen up to nine minutes; when the legs finally relaxed, they both dropped to the same extent. It is evident that sympathetic innervation is not essentially involved in the maintenance of tonic posture in the *toad*.

When the retracted deafferented leg is passively pulled down, it remains in this new extended position without resuming the previous flexion. No resistance is felt to the passive extension, such as would be found in the presence of proprioceptive innervation, mediating myotatic reflexes. After a leg has been partly or fully extended, it cannot be made to resume a more flexed position by passively flexing it. One might say, therefore, that this type of tonus exhibited by the deafferented limbs is plastic in one direction. According to Bremer and Moldaver (1934), tonic contractures appear in certain frog and toad muscles as after-discharges to reflex stimuli, even without proprioceptive reinforcement, but since these were only of several seconds' duration they can hardly be responsible for the prolonged contractures described above.

The conspicuous loss of tonus in the hind legs of *frogs* from which the sensory control has been removed either by deafferentation (Brondgeest, 1860; Cyon, 1874) or by peripheral anesthesia (v. Anrep, 1880), seems to contrast somewhat with the results described here for the *toad*. It must be borne in mind, however, that even in the frog great individual differences are found and that specimens have been reported which even after deafferentation exhibited a considerable residual tonus (Mommsen, 1885).

*Deafferentation of fore limbs.* Since the fore limbs in the toad are comparatively little concerned with locomotion, the examination of deafferented fore limbs offers less interest. Only for the sake of completeness, one case was studied.

The resting posture differs slightly from normal in that pronation and flexion of the shoulder and elbow are more accentuated.

Other than postural activities, however, have not been found to be markedly impaired by the deafferentation. Wiping toward the source of a stimulus, mechanical or chemical, applied to the head, is effected as in

normal animals, although with less vigor. The animal shows a tendency to use for the wiping the normal hind legs instead of the deafferented fore legs, and in order to bring about the reflex in the fore legs, the hind legs must be prevented from moving. This corresponds closely to what has been observed in deafferented mammalian fore limbs: The patterns for the coördinated use of the limb survive the deafferentation, but in the integrated activity of the animal these patterns are not activated as readily as in the presence of sensory control.

*Deafferentation of fore limbs and hind limbs.* Brown-Séquard (1850) has reported for the frog that completely deafferented animals are still capable of jumping and swimming. Since not much new light could be expected from a repetition of the experiments on the toad, only two animals of this type were studied. In both of these the spinal ganglia and dorsal roots of both fore limbs and both hind limbs were removed and the nerves of the adjacent segments cut. In one specimen all pairs of trunk nerves (5, 6, 7) were severed, in the other specimen only the seventh. After the operation both animals were extremely lethargic, scarcely showing any spontaneous actions. In this regard, their change from normal is immensely greater than one would expect from simply adding up the effects of fore limb and hind limb deafferentation. It is very difficult to induce these animals to move, but by strong and repeated olfactory or mechanical irritations eventually all the basic reactions described above for partially deafferented animals can be obtained, at least in traces. Jumping is rare, and if it occurs it is not effective. Occasionally, the animals move for a few steps with the hind legs alternating as in walking. From a source of ammonia odor they frequently retreat by pushing the body backward with the fore limbs alternating.

In contrast to the locomotor activities, which on the whole were extremely poor in these animals, some of the reflexes were executed with greater ease. Righting from the back occurred promptly to either side. Turn-table reflexes were as distinct as in other animals. Wiping reflexes to the head were occasionally obtained.

Again, we learn from this series of experiments that, however serious the effect of deafferentation may be with respect to the total behavior of the animals, it certainly cannot be said to involve a disintegration of the basic patterns of motor coördination as such, since, once made to appear, these manifest themselves in—qualitatively speaking—full integrity.

*Additional observations.* Some animals appeared to be perfectly normal immediately after coming out from the anesthesia, while others showed a marked weakness of the deafferented legs for hours. If persistent disturbances occur other than the minor ones described in this paper, one may take it for granted that the operation was improperly done, causing damage of some sort to the motor parts. As an immediate effect of the operation,

this was observed only in one of our animals in which the motor roots were injured in the resection of the ganglia. A marked stiffness in the legs of this animal caused a great awkwardness in jumping, walking and righting. A frequent extensor tetanus was characteristic of this specimen, reminding us of a similar phenomenon described by Brown-Séquard in the frog. Its absence in all the other specimens of our series indicates that where it happens it is not directly due to the deafferented condition but rather to motor damage of primary (operative) or secondary (degenerative) origin.

In two animals which had been quite normal after the operation, slight motor disturbances gradually developed during the following weeks. In one of these (bilaterally deafferented hind limbs) a certain weakness in the thighs appeared, in the other (unilateral deafferentation of hind limb) the deafferented limb began to lag occasionally behind the control limb during slow locomotion. Apart from these three cases, no distinct change was found in observations extended over a period of: up to one week in 18 cases, up to two weeks in 7 cases, and up to three weeks in 9 cases.

*B. Experiments on the axolotl.* The effects of deafferentation on motor function in urodele amphibia have, so far as I know, never before been tested. Spinal ganglia have been extirpated in adult newts (Locatelli, 1924), in order to study their influence on limb regeneration, but as this always involved the amputation of the limbs, no functional data were collected.

In the present work young axolotls (Amblystoma mexicanum) of about 5 to 7 cm. body length were used. The deafferentation, confined to the nerves innervating the left fore limb, consisted of the removal of the second, third, fourth and fifth ganglia and dorsal roots on the left side. Twelve animals were operated upon in this way. Most of them were afterwards used for limb transplantation experiments (P. Weiss, 1935a).

Physiological and histological checks were employed to ascertain that the operations had been successful in destroying the full sensory innervation of the limbs. In tests, regularly repeated over weeks after the operation, the deafferented limbs were found to be completely insensitive to mechanical stimulation. Not until many weeks or months after the operation did a certain amount of sensitivity reappear, due apparently to the ingrowth of collateral fibers from neighboring intact segments. The physiological tests were, furthermore, corroborated later by histological examination.

In these animals, the locomotor coördination of the deafferented fore limbs was, so far as could be seen, normal in every respect. Both in swimming and walking, forward and backward, the limb was moved in the typical manner, with the sole exception of one[5] case in which an almost

[5] By mistake two cases of this sort were reported in the preliminary communication.

complete paralysis of the limb had been caused by accidental injury to the motor roots at the time of the operation.

The resting posture of the deafferented limbs was completely normal in only four of the eleven functional cases. In the remaining seven cases, a certain hypertonicity of the adductor and extensor muscles was noticed, that is, the adduction and extension of the deafferented arm during rest was more accentuated than in the opposite normal arm. This difference, exhibited only occasionally by five of the animals, was a regular occurrence in two others. The individual variations, therefore, cover the whole range from an entirely normal posture on one end to a decidedly over-extended posture on the other. But even in the most pronounced cases, the abnormality disappeared at once with the beginning of locomotion. Then, the movements of the affected limb were not superimposed on a background of hyperextension, but were perfectly normal. As soon as the locomotor activity subsided, the hypertonicity of the adductors and extensors reappeared in the same degree as before, only to vanish again with the next movement. Even in those specimens in which the hypertonicity had initially been found, it gradually disappeared a few weeks later. The fact must be stressed that none of our animals showed a posture so strikingly abnormal as the one pictured by Detwiler and Vandyke (1934), nor was there any paralysis observed in our cases, except in the one with motor injury.

From our results we may conclude that the urodeles, as far as we can judge from the axolotl, their only representative examined, fall strictly in line with the anurans, frog and toad, in that the motor patterns of their limbs survive without disintegration the removal of their sensory innervation.

Discussion. The contention which is frequently heard that motor patterns are constructed, integrated and maintained by afferent excitations, concurring and interacting in the centers, implies that disintegration of the motor patterns must inevitably follow the elimination of those afferent excitations. We have furnished quite decisive proof that no such disintegration occurs. In the presence as well as in the absence of afferent impulses from a limb, the motor pattern, i.e., the coördinated activation of the individual muscles to perform a given movement, makes its appearance. Our experiments, extending the observations over long periods, within which residues of preceding afferent effects would necessarily have died away, have proved beyond doubt that the basic motor patterns can dispense with sensory control not only temporarily, but permanently, without being incapacitated.

The autonomy of central nervous activities is emphasized, in contrast to the view that central activity consists essentially in a mere through traffic of excitations released from the sensory and terminating in the motor

periphery. The inadequacy of this concept of mere uninterrupted reflex arcs has been strongly pointed out on anatomical grounds by Herrick (1930, 1934), while the autonomous character of central motor patterns, structural and dynamical, possibly activated by, but neither created nor maintained through, sensory influences, is a notion well in agreement with the anatomical realities, as well as with the results of our experiments.

On the other hand, it is plain that sensory control gains in significance as we move from the lower to the higher forms of vertebrates, until in man its importance may have become paramount. But while the higher vertebrates, when deafferented, suffer a loss of adjustability and precision in their finer movements, in the lowly amphibia no such finer movements on which the effects could become noticeable have yet been developed. It is not surprising, therefore, to find the deafferentation experiments on mammals and on amphibia yielding results which apparently conflict in a certain measure. But even this difference is not fundamental, as has been pointed out in the introductory remarks to this paper. The fishes fall in line with the amphibia, as demonstrated by a recent paper of Holst (1935) in which normal locomotion is described in fishes with all dorsal roots destroyed bilaterally.

If our experiments are conclusive in demonstrating the persistence of motor patterns after deafferentation, they do not, at the same time, definitely exclude the possibility that motor patterns owe their original existence to sensory influences, as claimed by Detwiler and Vandyke. Our work, dealing only with established and not with developing motor patterns, cannot directly invalidate their contention; but it makes it appear highly improbable, and in this respect concurs with evidence gained from other sources: One remembers the experiments in which development of motor functions has been secured in the absence of sensory control, by rearing amphibian larvae in permanent anesthesia from pre-functional embryonic stages on (Harrison, 1904; Carmichael, 1926; Matthews and Detwiler, 1926). In appraising the case, it should be borne in mind that Detwiler and Vandyke have not been able to remove purely and exclusively the sensory innervation; the ablation of the dorsal part of the neural tube, as executed in their experiments, entails, besides a suppression of the formation of spinal ganglia, an alteration of the development of the spinal cord. Detwiler and Vandyke, comparing the functional deficiencies in their cases with both the degree of sensory defects and the amount of abnormality found in the quantitative development of the spinal cord, assert that the functional defects reflect the ganglionic rather than the intraspinal defects. But the weak point in this argument is that defects of the spinal cord are compared on a merely quantitative and numerical basis, disregarding the possibility that intracentral defects of the same extent may be of entirely different seriousness depending on the particular part they involve.

Another attempt to demonstrate the indispensability of sensory, in particular proprioceptive, excitations for the development as well as for the performance of coördinated motor functions has recently been made by Nicholas and Barron (1935). In Amblystoma they examined the response of a fore leg to electrical stimulation of contralateral dorsal roots, responses which they contend to be crossed reflexes. Two main results were reported: 1. The crossed response is obtained only in those cases where the stimulated root has previously been in actual connection with a limb. This is interpreted as evidence that the intracentral reflex pattern requires for its establishment the presence of afferent fibers actually connected with a limb. 2. Stimulation of any one dorsal root of the limb plexus yields responses of the ipsilateral or contralateral limb only provided these limbs possess motor connections with the very same spinal segment into which the stimulated root discharges. It is claimed, therefore, that the intracentral effects of each dorsal root are strictly confined to those motor roots which emerge from its own segment, without reaching neighboring segments. This claim, being in obvious contradiction to a host of facts of reflex physiology, naturally arouses the closest scrutiny. Countless examples have been furnished by Sherrington's work (1906, 1929), showing that excitations entering the spinal cord in different segments affect the same "final common paths." Brücke's (1922) demonstration of the central interference of excitations set up by series of stimuli of different frequency applied to different segmental nerves in the frog offers another illuminating example.

As an additional test, I have recently undertaken a few experiments in which reflex responses were recorded by action potential registration (amplifier and Matthews oscillograph) from the motor nerves.[6] In curarized frogs, one of the hind limb nerves (8, 9, 10), all of which had been severed from their peripheral connections, was stimulated with a series of condenser discharges, while the action potentials were recorded successively from the three contralateral limb nerves, likewise severed distally. It was found, as expected, that when a single nerve was stimulated, series of action potentials, indicating reflex discharges, appeared in all three contralateral limb nerves, in animals with intact brain as well as in decerebrated animals. There was no indication of a restriction of the response to the stimulated segment, such as has been suggested by Nicholas and Barron.

A critical inspection of the data presented by Nicholas and Barron reveals that these workers must have been dealing with direct responses of

[6] These experiments were done in collaboration with Dr. R. J. Pumphrey of the University of Cambridge (England) in the laboratory of Dr. D. W. Bronk in the Eldridge Reeves Johnson Foundation for Medical Physics, University of Pennsylvania. The author is greatly indebted to Doctor Bronk and Doctor Pumphrey for their valuable help. The work was aided through a grant received from the General Education Board.

muscles and nerves to the stimulating current rather than with reflex responses. This can be inferred from the fact that all the responses described as reflex in nature by the authors, were obtained while the animals were in a state of central narcosis wh ch in these forms is well known to abolish spinal reflexes altogether. Furthermore, the method of stimulation, employed by the authors, was not such as to permit the application of strictly localized stimuli. Apparently neither the stimulating electrode (unipolar stimulation) nor the stimulated root were sufficiently insulated against the surrounding tissue; hence, the current passing through the animal and converging upon the "different" electrode along undefinable lines, could elicit contralateral nerve and muscle responses despite the narcosis of the reflex apparatus. The peculiar type of the responses obtained is indicative of the particular pattern of the lines of current rather than of the pattern of the reflex mechanism. Based as they are on a deceptive method, the conclusions of the authors in regard to the rôle of dorsal root fibers in the development of motor patterns cannot, therefore, be accepted until they have been supplemented by more adequate proof.

## SUMMARY

Spinal ganglia and dorsal roots were excised from the limb region of toads and axolotls, and the motor functions of the deafferented limbs were studied from the time immediately after the operation up to several weeks later.

*Toads.* Locomotion (jumping, walking, swimming) and reflexes (righting, turning, wiping) were not noticeably impaired by the unilateral or bilateral deafferentation of either the hind limbs or the fore limbs. Even completely deafferented animals, although very inactive, display coördinated movements.

Strong tonic flexion can be maintained for several minutes in deafferented hind limbs, as in normal limbs, when the animals are vertically suspended. Sympathectomy combined with the deafferentation has no visible effect on this tonic capacity.

*Axolotl.* The locomotor coördination of deafferented fore limbs was found to be perfectly normal. In some animals a slight hyperextension characterized the resting posture.

All the experiments described emphasize the central autonomy of motor patterns and their wide independence of proprioceptive control in Amphibia.

## REFERENCES

VON ANREP, B. Pflüger's Arch. **21:** 226, 1880.
BALDI, D. Sperim. Giorn. Ital. di Scienze Mediche **56:** 265, 1885.
BELL, C. Philosoph. Trans. Roy. Soc. London, 1821, p. 398.
BERNARD, C. Compt. rend. Soc. Biol. **1:** 15, 1850.

BICKEL, A. Pflüger's Arch. **67:** 299, 1897.
DE BOER, S. Folia Neurobiol. **7:** 378, 1913.
BREMER, F. Ergebn. Physiol. **34:** 678, 1932.
BREMER, F. ET J. MOLDAVER. C. v. Soc. Biol. **117:** 821, 1934.
BRONDGEEST, P. J. Arch. f. Anat., Physiol. u. wiss. Med. 1860, p. 703.
BROWN, J. G. J. Physiol. **48:** 18, 1914.
BROWN-SÉQUARD. Compt. rend. Soc. Biol. **1:** 15, 1850.
v. BRÜCKE, E. T. Ztschr. f. Biol. **77:** 29, 1922.
CARMICHAEL, L. Psychol. Rev. **33:** 51, 1926.
COBB, S. AND H. G. WOLFF. Arch. Neurol. and Psychiat. **28:** 661, 1932.
CYON, E. Pflüger's Arch. **8:** 347, 1874.
DETWILER, S. R. Biol. Rev. **8:** 269, 1933.
DETWILER, S. R. AND R. H. VANDYKE. J. Exper. Zool. **68:** 321, 1934.
EXNER, S. Zentralbl. Physiol. **3:** 115, 1890.
Entwurf zu einer physiologischen Erklärung der psychischen Erscheinungen. Leipzig u. Wien. 1894.
FILEHNE, W. Arch. Anat. u. Physiol. Physiol. Abtlg. S. 432, 1886.
HARRISON, R. G. Am. J. Anat. **3:** 197, 1904.
Am. J. Anat. **5:** 121, 1906.
J. Comp. Neurol. **37:** 123, 1924.
HERING, H. E. Pflüger's Arch. **54:** 614, 1893.
Arch. exper. Path. u. Pharmakol. **38:** 266, 1896.
Pflüger's Arch. **68:** 1, 1897.
Neurol. Zentralbl. **16:** 1077, 1897.
HERRICK, C. J. Proc. Nat. Ac. Sc. **16:** 643, 1930.
J. Comp. Neurol. **59:** 239, 1934.
v. HOLST, E. Pflüger's Arch. **235:** 345, 1935.
LOCATELLI, P. Arch. di Scienze biol. **5:** 362, 1924.
MATTHEWS, S. C. AND S. R. DETWILER. J. Exper. Zool. **45:** 279, 1926.
MERZBACHER, L. Pflüger's Arch. **88:** 453, 1902.
Pflüger's Arch. **92:** 585, 1902a.
MOMMSEN, J. Virchow's Arch. **101:** 22, 1885.
MOSSO, A. Arch. Ital. de Biol. **41:** 183, 1904.
MOTT, F. W. AND C. J. SHERRINGTON. Proc. Roy. Soc. London **57:** 481, 1895.
MUNK, H. Sitzgber. Preuss. Ak. Wiss. 1903, S. 1038.
NICHOLAS, J. S. AND D. H. BARRON. J. Comp. Neurol. **61:** 413, 1935.
PANIZZA. Ricerche sperimentali sui nervi. Pavia 1837.
PINELES, F. Zentralbl. Physiol. **4:** 741, 1891.
RANSON, S. W. AND J. C. HINSEY. J. Comp. Neurol. **42:** 69, 1926.
RANSON, S. W. Arch. Neurol. and Psych. **19:** 201, 1928.
SCHÖPS. Meckel's Arch. 1824, S. 409.
SHERRINGTON, C. J. The integrative action of the nervous system. 1906.
Proc. Roy. Soc. London, Ser. B. **105:** 332, 1929.
STILLING, B. Arch. f. physiol. Heilkunde, p. 97, 1842.
TRENDELENBURG, W. Arch. Anat. u. Physiol. Physiol. Abtlg. 1906, S. 1.
WEISS, P. Anat. Rec. **60:** 437, 1934.
J. Comp. Neurol. **61:** 135, 1935.
Proc. Soc. Exper. Biol. and Med. **33:** 30, 1935.

# THE CHLORIDE AND TOTAL BASE CONTENTS OF TENDON AND CARTILAGE

MARY WHELAN AND H. A. SHOEMAKER

*From the Department of Pharmacology, University of Oklahoma, School of Medicine, Oklahoma City*

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During the course of work on the distribution of chlorides and iodides in tissues of rabbits after the administration of potassium iodide, it was observed that the concentration of these elements was considerably higher in the muscle of the abdomen than in that of the back (9). The abdominal muscle has proportionately more connective tissue. A search of the literature did not give much information on the chloride content of connective tissue. Bunge (2) determined the chloride, phosphorus, water and cation content of cartilage of the shark; and Cameron and Walton (3) the chloride content of tendon of dogs. Close (4) gives the chloride content of human tissues as determined in *post-mortem* material. Because of this scarcity of data, determinations of chloride and total base content of certain tissues of dogs, cats and rabbits were undertaken. Particular attention was given to cartilage and tendon, and for purposes of comparison, analyses of muscle and skin were also made.

PROCEDURE. Dogs, rabbits and cats were used in these studies. Food and water were withheld for a period of twelve hours before death. The rabbits were stunned by a blow on the head and then bled as completely as possible by cutting the carotids. The dogs and cats were anesthetized with ether and then bled. After removal of hair with fine clippers portions of the skin were removed for analysis and freed, as far as possible, from fat and subcutaneous tissue. Muscle from the back and the abdomen was separated from the sheath and a portion removed. In the case of the rabbit the whole abdominal wall was used for analysis. The cartilage of the ear was freed by stripping off the skin. Through the courtesy of the Department of Pathology of the University Hospital, human skin, muscle and tendon was secured from a seventeen year old boy's leg which had been amputated because of an osteogenic sarcoma of the tibia.

All tissues were ground in a meat chopper with a fine knife. Fresh tissue was used and all analyses made in duplicate. Chlorides were determined by the method of Wilson and Ball (12), and total base by a slightly modified method of Stadie and Ross (10). The moist tissue was

TABLE 1

*Chloride and total base in 100 grams fresh tissue*

| AUTHOR | ANIMAL | TISSUE | TOTAL BASE | CHLORIDE | CHLORIDE |
|---|---|---|---|---|---|
| | | | *cc. N 10* | *cc. N 10* | *mgm.* |
| Bunge (1) | Cow | Muscle | 144.3 | 18.9 | 67.2 |
| Bunge (2) | Shark | Cartilage | 230.5 | 136.0 | 483.2 |
| Katz* (7) | | Muscle | 90.5 | 9.0 | 32.0 |
| Katz* (7) | | Muscle | 233.3 | 22.5 | 80.0 |
| Magnus-Levy (8) | Human | Muscle | | 17.2 | 61.0 |
| Magnus-Levy (8) | Steer | Tunica Albuginea | | 93.6 | 332.0 |
| Damiens (6) | Dog | Trachea | | 22.6 | 80.0 |
| Damiens (6) | Dog | Muscle | | 15.5 | 55.0 |
| Vladesco (11) | Cow | Ileo-spinal muscle | | 24.1 | 85.7 |
| Vladesco (11) | Cow | Right internal muscle | | 29.4 | 104.4 |
| Cameron and Walton (3) | Dog | Tendon | | 73.5 | 261.0 |
| Cameron and Walton (3) | Rat | Muscle | | 16.9 | 60.0 |
| Close (4) | Human | Tendo Achilles | | 62.0 | 220.0 |
| | | Connective tissue | | 56.4 | 200.0 |
| | | Cartilage (articular) | | 36.6 | 130.0 |
| | | Skin | | 62.0 | 220.0 |
| | | Muscle | | 22.6 | 80.0 |
| Cullen et al. (5) | Human | Gastrocnemius muscle | 110.0 | 35.8 | 127.0 |

* Data compiled from analyses of large number of animals—minimum and maximum values are given.

TABLE 2

*Chloride—milligrams per 100 gram tissue*

| | DOG 1 | DOG 2 | DOG 3 | AVERAGE | CAT 1 | CAT 2 | AVERAGE | RABBIT 1 | RABBIT 2 | RABBIT 3 | AVERAGE | HUMAN |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Muscle of abdomen | | 66 | 85 | 71 | 72 | 72 | 72 | 65 | 69 | 64 | 66 | |
| Muscles of back | | 43 | 61 | 52 | 24 | 36 | 30 | 31 | 39 | 32 | 34 | 60* |
| Trachea | 169 | 182 | 214 | 188 | 213 | 270 | 241 | 26 | 72 | 44 | 47 | |
| Tendon | 212 | 292 | 288 | 264 | 282 | 276 | 279 | 264 | 210 | 203 | 226 | 248 |
| Cartilage of ear | 212 | 215 | 285 | 237 | | 275 | | 216 | 182 | 187 | 195 | |
| Skin | 196 | 222 | 199 | 206 | 219 | 246 | 236 | 214 | 213 | 223 | 217 | 204 |

* Muscle of leg.

weighed in a porcelain crucible, a few drops of concentrated sulphuric acid added and then heated on an electric plate (low temperature) until the tissue was quite well destroyed. The crucibles were then placed in a muffle furnace and allowed to remain over night at a temperature not exceeding 500°C. A white ash readily soluble in 0.25 cc. concentrated sulphuric acid was obtained. The sulphates were then quantitatively transferred, by washing with approximately 10 cc. of distilled water, to a test tube graduated at 25 cc. From this point the procedure was that of Stadie and Ross (10).

DISCUSSION. In table 1 the results of analyses of fresh tissues are assembled from the literature. The figures for total base were computed from determinations of sodium, potassium, calcium and magnesium content of muscle and cartilage.

TABLE 3

*Total base—cubic centimeters N/10 per 100 gram tissue*

| | DOG 1 | DOG 2 | DOG 3 | AVERAGE | CAT 1 | CAT 2 | AVERAGE | RABBIT 1 | RABBIT 2 | RABBIT 3 | AVERAGE | HUMAN |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Muscle of abdomen | 116 | 120 | 125 | 120 | 157 | 98 | 128 | 108 | 109 | 119 | 112 | |
| Muscle of back | 116 | 120 | 108 | 115 | 130 | 87 | 108 | 114 | 90 | 84 | 96 | 111* |
| Trachea | | 359 | 259 | 306 | | | | | | | | |
| Tendon | 110 | 155 | 121 | 129 | 148 | 110 | 129 | 128 | 119 | 122 | 123 | 102 |
| Cartilage of ear | 209 | 261 | 262 | 244 | | 194 | | 212 | 219 | 208 | 213 | |
| Skin | 96 | 127 | 100 | 108 | 78 | 103 | 140 | 133 | 136 | 123 | 131 | 88 |

* Muscle of leg.

In tables 2 and 3 are listed the results of our analyses for chloride and total base values of the same tissue in the same species as well as in the different species of animals. The chloride content of tissues containing a large amount of connective tissue is always high.

## SUMMARY

Data for chloride and total base contents of muscle, skin, tendon, and cartilage from various sources have been presented.

## REFERENCES

(1) BUNGE, G. Ztschr. physiol. Chem. **9:** 60, 1885.
(2) BUNGE, G. Ztschr. physiol. Chem. **28:** 300, 1889.
(3) CAMERON, A. T. AND C. H. A. WALTON. Trans. Roy. Soc. Can., **22:** Sec. r, 1928; Abs., Chem. Abs., **23:** 157; 1928.
(4) CLOSE, H. G. Biochem. J. **27:** 967, 1933.

(5) CULLEN, G. E., W. E. WILKINS AND T. R. HARRISON. J. Biol. Chem. **102:** 415, 1933.
(6) DAMIENS, M. A. Compt. Rend. **171:** 930, 1920.
(7) KATZ, J. Pflüger's Arch. **63:** 1; Abs. J. Chem. Soc. **70:** 377, 1896.
(8) MAGNUS-LEVY, A. Biochem. Ztschr. **24:** 363, 1910.
(9) SHOEMAKER, H. A. AND F. P. UNDERHILL. J. Pharmacol. and Exper. Therap. **44:** 23, 1932.
(10) STADIE, W. C. AND E. C. ROSS. J. Biol. Chem. **65:** 735, 1925.
(11) VLADESCO, R. Bull. Soc. Chem. Romenia **7:** 29; Abs., Chem. Abs. **23:** 157, 1925.
(12) WILSON, D. W. AND E. G. BALL. J. Biol. Chem. **79:** 221, 1928.

# THE RELATION OF PREGNANCY AND LACTATION TO EXTIRPATION DIABETES IN THE DOG

F. P. CUTHBERT, A. C. IVY, B. L. ISAACS AND JOHN GRAY

*From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago*

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The literature on the effect of pregnancy on the severity of the diabetic condition is discordant. Because we suspected that the discordance might be due to certain differences in experimental approach, our investigation was undertaken.

Carlson and his collaborators (1–3) observed that hyperglycemia and glycosuria did not result or was slight following pancreatectomy in gravid dogs near term. This was confirmed on one dog each by two observers (4, 5). On the contrary, Allen (6) failed to observe that pregnancy had an effect on partially depancreatized dogs, and Markowitz and Simpson (7, 8) observed no change in carbohydrate metabolism during pregnancy in a depancreatized dog (inadequately studied) receiving adequate doses of insulin. However, the latter observers reported the occurrence of hypoglycemic symptoms after parturition in their dog, which was attributed to the formation of milk. Later, Markowitz and Soskin (9) studied another depancreatized pregnant dog, which received insulin in doses just inadequate to prevent glycosuria, and observed no significant change in sugar excretion as pregnancy advanced. They also repeated the work of Carlson on two dogs, with the exception that they administered insulin in small (3–5 u. daily) doses, and failed to obtain evidence confirmatory of Carlson's results.

A review of the "post-insulin" clinical literature on this subject reveals that some (10–14) report that pregnancy intensifies and others (15–22) that it ameliorates the diabetes. Most agree that the state of early pregnancy intensifies or does not change the diabetic condition and that lactation ameliorates it. Kramer (23) recently on reviewing one hundred and ten cases in the literature, found that in late pregnancy the sugar tolerance was impaired in 29 per cent, unchanged in 24 and improved in 47 per cent of patients. Hypertrophic and hyperplastic islets have been observed in several infants born of diabetic mothers (24, 25, 34, 35) and hypoglycemic symptoms in such infants have also been observed.

METHODS IN GENERAL. We have performed three types of experiments.

*Experiment I.* In the first experiment we depancreatized two female dogs and gave them a uniform maintenance diet and a quantity of insulin which permitted the excretion of a small amount of sugar daily. One of the dogs was then followed through two gestation and lactation periods, and one through one gestation and lactation period. *Experiment II.* In the second experiment, we repeated the procedure of Carlson in which pregnant dogs near term were depancreatized and given no insulin. *Experiment III.* Then, we repeated the experiment of Markowitz and Soskin (9) in which the dogs were depancreatized near term and given 3 u. of insulin twice daily. Autopsy was performed on all dogs, and examination made for pancreatic remnants and other changes.

*Experiment I. Methods:* After pancreatectomy (ether morphine anesthesia) two accurately weighed meals were fed at the same hour each day, insulin being injected just before the animal started to eat. Minced fresh calves' pancreas (200 grams) was added to the basal diet (754 cal.), which contained lean cooked meat (200 grams) and milk (250 cc.). This constituted the daily ration. The diet remained the same throughout the period of the experiment, for almost three years, and the dogs consumed all their food without exception. The insulin dosage was held constant except when it was desirable to change it because of the absence of glycosuria or to determine the insulin-glucose equivalent. The necessary precautions were taken to insure that variations in insulin requirement were not due to differences in potency of the insulin used. The urine was collected twice daily (25), care being taken to prevent bacterial action. Blood sugar determinations were made only occasionally to check the absence of glycosuria.

The theoretical dextrose yield of the diet was approximately 60 grams, all except 12.8 grams (milk) being calculated as originating from protein and fat. The actual yield in these diabetic dogs ranged from 45 to 55 grams of sugar when given no insulin. If the carbohydrate content of the diet had been higher, the insulin-glucose equivalent shown in the tables 1, 2 and 3 would have been greater perhaps (26). Markowitz and Soskin (9) added 40 grams of sucrose to their diet, which otherwise was like ours. We chose a low carbohydrate diet, because we thought that on such a diet the maternal metabolism might be a more sensitive indicator of any influence the products of conception might have (26).

*Results, experiment I.* The essential data are given in tables 1, 2 and 3. The daily data are not included, except as indicated, since the dogs were studied for a period of about three years. Of course, the daily excretion of sugar sometimes varies as much as fifty per cent, because the dogs were not catheterized; but the five day averages are fairly constant. Dog 2 "missed" one pregnancy, which is the reason why we do not have data from two pregnancies on this dog. It should be noted that the dogs remained in excellent

condition at all times. They gained weight during each pregnancy, but lost during lactation, as might be expected.

During estrus and early gestation, sugar excretion was somewhat augmented. In all three instances, the sugar excretion diminished during the latter week or twelve days of gestation. The insulin requirement, as indicated by glycosuria, was also diminished, approximately 33 per cent in first pregnancies of both dogs and about 22 per cent in the second of dog 1.

TABLE 1

*Dog 1. First pregnancy and lactation period. Dog was operated and "standardized" for four months prior to April 10. Diet constant*

| DATE | AVERAGE DAILY SUGAR EXCRETION | BODY WT. | UNITS INSULIN DOSAGE | INSULIN-GLUCOSE EQUIVALENT | DATE | AVERAGE DAILY SUGAR EXCRETION | BODY WT. | UNITS INSULIN DOSAGE | INSULIN-GLUCOSE EQUIVALENT |
|---|---|---|---|---|---|---|---|---|---|
| | *gm.* | *lbs.* | | | | *gm.* | *lbs.* | | |
| April 10–May 12 | 2.10 | 17 | 40 | 1.45 | Sept. 10–17 | 1.59 | 14.5 | 16 | |
| | | | | | Sept. 17–24 | 1.66 | | 16 | |
| May 12–22 | 12.71 | 17 | 30 | 1.58 | Sept. 24–27 | 2.73 | 15.0 | 16 | 3.2 |
| May 22–29 | 12.93 | 17 | 30 | 1.56 | Sept. 27–Oct. 19 | 2.15 | 15.5 | 16 | |
| May 29–June 5 | 16.21 | 17 | 30 | 1.46 | | | | | |
| | Estrus | | | | Oct. 19–Nov. 23 | 4.48 | 15.5 | 16 | 3.4 |
| June 5–12 | 13.02 | | 30 | | | | | | |
| | Copulating | | | | Nov. 23–Dec. 24 | 7.26 | | 16 | |
| June 12–19 | 12.39 | | 30 | | | | | | |
| June 19–26 | 11.41 | | 30 | 1.6 | Dec. 24–Jan. 13 | 14.10 | | 16 | 2.7 |
| June 26–July 3 | 5.60 | 17.7 | 30 | 1.8 | | | | | |
| July 3–10 | 10.01 | | 30 | 1.6 | Jan. 13–31 | 7.63 | 16.0 | 20 | |
| July 10–17 | 5.35 | 18.2 | 30 | 1.79 | Jan. 31–Feb. 19 | 3.00 | 17.0 | 20 | 2.7 |
| July 17–22 | 6.98 | | 30 | | | | | | |
| July 22–29 | 6.85 | 19.0 | 25 | 2.15 | Feb. 19–25 | 9.34 | | 18 | 2.8 |
| July 29 | | | | | Feb. 25–28 | 4.95 | | 20 | 2.7 |
| Aug. 5 | 5.33 | 20.2 | 25 | | Feb. 28–Mar. 23 | 6.74 | 17.5 | 18 | |
| Aug. 5–11 | 0.91 | 20.5 | 20 | 3.0 | | | | | 2.8 |
| Aug. 11 | 5 full-term living pups born | | | | Mar. 23–April 23 | 9.61 | | 18 | |
| Aug. 12 | 20.85 | 17.5 | 20 | | | | | | |
| | Ate placentae | | | 2.0 | | | | | |
| Aug. 13 | 16.9 | | 20 | | | | | | |
| Aug. 13–20 | 1.62 | 16.75 | 20 | | | | | | |
| Aug. 20–27 | 1.74 | 15.5 | 16 | | | | | | |
| Aug. 27–Sept. 3 | 2.79 | 15.5 | 16 | 3.6 | | | | | |
| Sept. 3–10 | 1.25 | | 16 | | | | | | |
| | Pups removed | | | | | | | | |

During the day following delivery, and in two instances during the second day, also, the sugar excretion rose quite definitely, to fall again as milk secretion started. Reduction of the insulin dosage at some part of the lactation period was required in all cases. When the pups were removed, the sugar excretion rose promptly in two of the three instances. The first pregnancy of dog 1 was the exception. It is to be noted that this dog initially required 40 u. of insulin to keep the average daily excretion of sugar

TABLE 2

*Dog 1. Second pregnancy and lactation period. Diet constant*

| DATE | AVERAGE DAILY SUGAR EXCRETION | BODY WT. | UNITS INSULIN DOSAGE | INSULIN-GLUCOSE EQUIVALENT | DATE | AVERAGE DAILY SUGAR EXCRETION | BODY WT. | UNITS INSULIN DOSAGE | INSULIN-GLUCOSE EQUIVALENT |
|---|---|---|---|---|---|---|---|---|---|
| | *gm.* | *lbs.* | | | | *gm.* | *lbs.* | | |
| April 23–30 | 6 81 | 17 5 | 18 | | Aug. 2–5 | 2 54 | 19 5 | 14 | 4 1 |
| April 30–May 6 | 7 46 | | 18 | 2 9 | Aug. 5 | 4 full term living pups born | | | |
| May 6–13 | 9 19 | | 18 | | Aug. 6 | 15 65 | | 14 | 3 1 |
| May 13–20 | 10 46 | | 18 | | | Ate placentae | | | |
| | Estrus | | | | Aug. 7 | 9 47 | 16 5 | 14 | 3 6 |
| May 20–27 | 13 14 | | 18 | 2 6 | | 2 pups died | | | |
| | Copulating | | | | Aug. 8 | 13 7 | | 14 | |
| May 27–June 3 | 16 81 | | 18 | | Aug. 9–16 | 11 49 | | 14 | 3 38 |
| June 3–10 | 13 79 | 18 0 | 18 | | | 1 pup died | | | |
| June 10–17 | 13 51 | | 18 | 2 58 | Aug. 16–23 | 12 12 | 16 2 | 14 | |
| June 17–24 | 13 31 | | 18 | | Aug. 23–30 | 8 77 | | 14 | 3 65 |
| June 24–July 1 | 15 68 | 1 | 18 | 2 46 | Aug. 30 to Sept. 4 | 3 28 | | 14 | 4 0 |
| July 1–8 | 18 21 | 18 2 | 18 | 2 35 | Sept. 4–7 | 0 00 | | 12 | 5 0 |
| July 8–15 | 13 61 | | 18 | 2 57 | | Very large pup removed | | | |
| July 15–22 | 8 84 | | 18 | | Sept. 7–13 | 13 17 | 14 2 | 10 | 4 68 |
| July 22–29 | 4 84 | 19 2 | 18 | | Sept. 13–20 | 10 64 | | 14 | 3 5 |
| July 29–Aug. 2 | 0 44 | | 18 | 3 3 | Sept. 20–24 | 18 1 | 16 0 | 14 | 2 9 |
| | | | | | Sept. 24–Oct. 1 | 11 02 | | 18 | 2 7 |
| | | | | | Oct. 1–8 | 12 01 | | 18 | |
| | | | | | Oct. 8–April 25 (next) | 8 82 | 17 0 | 20 | 2 56±0 2 |

TABLE 3

*Dog 2. One pregnancy and lactation period, the other pregnancy being "missed." Dog was operated and "standardized" for more than 12 months prior to April 21. Diet constant*

| DATE | AVERAGE DAILY SUGAR EXCRETION | BODY WT. | UNITS INSULIN DOSAGE | INSULIN-GLUCOSE EQUIVALENT | DATE | AVERAGE DAILY SUGAR EXCRETION | BODY WT. | UNITS INSULIN DOSAGE | INSULIN-GLUCOSE EQUIVALENT |
|---|---|---|---|---|---|---|---|---|---|
| | *gm.* | *lbs.* | | | | *gm.* | *lbs.* | | |
| Feb. 10–26 | 8 16 | 22 0 | 22 | 2 36 | July 28–Aug. 4 | 4 71 | | 10 | |
| Feb. 26–May 19 | 12 43 | 22 0 | 19 | 2 52 | Aug. 4–11 | 6 29 | 18 5 | 10 | 5 5 |
| | | | | | Aug. 11–18 | 5 13 | | 10 | |
| May–Oct. | 6 66 | 22 0 | 22 | 2 4 | Aug. 18–22 | 3 18 | | 10 | |
| Oct.–April | 8 63 | 22 2 | 22 | 2 4 | Aug. 22–29 | 9 91 | | 10 | 5 0 |
| April 21–28 | 7 66 | 22 5 | 22 | 2 38 | | Removed pups | | | |
| April 28–May 5 | 7 87 | | 22 | | Aug. 29–Sept. 6 | 13 06 | | 10 | 4 7 |
| May 5–12 | 10 32 | | 22 | 2 26 | | | | | |
| May 12–19 | 8 66 | 22 5 | 22 | 2 4 | Sept. 6–13 | 22 02 | 18 0 | 10 | 3 8 |
| May 19–26 | 13 72 | | 22 | 2 1 | Sept. 13–17 | 22 6 | | 10 | 3 74 |
| | Copulating | | | | Sept. 17–25 | 14 09 | 18 0 | 14 | 3 28 |
| May 26–June 2 | 18 47 | | 22 | 1 88 | Sept. 25–Oct. 2 | 4 31 | | 18 | 3 12 |
| June 2–9 | 14 12 | 23 0 | 22 | | Oct. 2–9 | 3 28 | | 18 | |
| June 9–16 | 16 13 | | 22 | | Oct. 9–18 | 16 08 | 19 5 | 18 | 2 44 |
| June 16–23 | 14 11 | | 22 | | Oct. 18–Nov. 1 | 10 7 | | 20 | 2 45 |
| June 23–30 | 15 41 | | 22 | 2 0 | Nov. 1–10 | 11 74 | | 22 | 2 18 |
| June 30–July 7 | 15 65 | | 22 | | Nov. 10–20 | 11 05 | | 22 | 2 22 |
| July 7–14 | 5 44 | 24 0 | 22 | 2 48 | Nov. 20–30 | 3 59 | 22 0 | 22 | 2 54 |
| July 14–19 | None | | 22 | | Nov. 30–Dec. 8 | 3 64 | | 22 | 2 41 |
| July 19–21 | None | | 20 | | Dec.–May (next) | 7 46 | 22 0 | 22 | 2 38 |
| July 21–23 | None | | 18 | | | | | | |
| July 23–25 | None | 26 25 | 14 | 4 28+ | | | | | |
| July 25 | 5 full term living pups born | | | | | | | | |
| July 26 | 13 15 | 22 75 | 10 | | | | | | |
| | Ate placentae | | | 4 63 | | | | | |
| July 27 | 14 3 | | 10 | | | | | | |

at a level of from 2 to 4 grams. After the first pregnancy, the requirement was only from 18 to 20 units, to which value it again returned after the second pregnancy. We cannot account for this fifty per cent reduction, no detectable infectious process being present before the first pregnancy.

Table 4 gives the essential data pertaining to the changes in the insulin-glucose equivalents for the more important periods. It is to be noted that the trend is the same in the three gestation and lactation periods and that the values for the second pregnancy of dog 1 check closely with those for the pregnancy of dog 2.

*Discussion of results of experiment 1.* The decrease in sugar excretion is quite evident during the latter part of pregnancy and lactation. We believe the decrease is significant, because we have found in this and other work (27), like Allan (26) and others, that with a constant diet and insulin dosage and the animal in good condition, the insulin-glucose equivalent is quite constant. The change observed in dog 1 after the first gestation and lactation period is the only unaccounted for change that we have

TABLE 4

*The insulin-glucose equivalent at different periods*

| DOG | BEFORE PREG. | EARLY PREG. | JUST BEFORE DELIVERY | DAY AFTER DELIVERY | DURING LACTATION | AFTER LACTATION | LATER |
|---|---|---|---|---|---|---|---|
| Dog 1 1st | 1.5 | 1.6 | 3.0 | 2.0 | 3.6 | 3.2 | 2.7 |
| Dog 1 2nd | 2.9 | 2.3 | 4.1 | 3.1 | 5.0 | 2.9 | 2.7 |
| | | | | | | | 2.6 |
| Dog 2 | 2.4 | 1.9 | 4.28+ | 4.63 | 5.5 | 3.0 | 2.4 |

observed in six dogs receiving raw pancreas and kept for one year or longer. The increase in the insulin-glucose equivalent during late gestation is also significant, but is not to be emphasized because the equivalent is frequently elevated some whenever the dosage of insulin is decreased (26); this is truer when a high carbohydrate diet is fed.

The increase in glycosuria following parturition for a day or two may indicate that fetal insulin was passing into the maternal circulation. However, it should be pointed out that the mother ingested the placentae.

Our failure to confirm the observation of Markowitz and Soskin (9) may be due either to the low carbohydrate diet we fed purposely, or to the possibility that the metabolism of individual depancreatized dogs reacts differently to pregnancy as diabetic human mothers apparently do.

These observations obviously do not demonstrate that fetal insulin entered the maternal circulation. No one has actually made such a demonstration. However, under anesthesia the fetuses have been injected with insulin by Pack and Barber (24), who used the goat, by Olow

(28) Naeslund (31) and Rupp (29), who used the rabbit, by Corey (32) who used the rat, and by Schlossmann (30), who used the dog and cat. It was found by all that the maternal blood sugar falls, although Snyder

TABLE 5

*Dog 4. Depancreatized almost 4½ days without showing diabetes. Seven living mature pups born*

| DATE | HOUR | BLOOD SUGAR | URINE VOLUME | URINE % SUGAR | TOTAL SUGAR | REMARKS |
|---|---|---|---|---|---|---|
| 6/18 | Noon | 92 | | | | Weight = 16 kgm. |
| 6/19 | 8 a.m. | 84 | 300 | None | | |
| | 10 a.m. | Depancreatized | | | | |
| | 6 p.m. | 95 | | | | |
| | 10 p.m. | 101 | | | | |
| 6/20 | 8 a.m. | 89 | 520 | None | | 1000 cc. 0.9% Cl subcut. |
| | 5 p.m. | 64 | | | | Ate 500 cc. milk |
| | 11 p.m. | 188 | | | | |
| 6/21 | 6 a.m. | 130 | 1200 | None | | Ate 250 cc. milk |
| | 12 a.m. | 158 | | | | Ate 150 cc. milk |
| | 5 p.m. | 135 | | | | Ate 200 cc. milk |
| 6/22 | 9 a.m. | 77 | 800 | None | | |
| | 2 p.m. | | | | | 200 gm. meat with 200 cc. milk. Blood total fatty acid—508. Total cholesterol—180. Free cholesterol—65 |
| | 5 p.m. | 225 | | | | Postcibum hyperglycemia |
| 6/23 | 9 a.m. | 88 | 900 | Trace* | | 250 cc. milk, 200 gm. meat |
| | 3 p.m. | 176 | | | | |
| 6/24 | 9 a.m. | 251 | 600 | Trace* | | 6 living mature pups, delivered during night |
| | 10 a.m. | | | | | Ate 250 cc. milk, 200 gm. meat, 100 gm. pancreas |
| | 5 p.m. | 405 | | | | Refused food |
| 6/25 | 8 a.m. | | 1850 | 3.0 | 55.5 | 7th pup delivered, living. No lactation |
| | 6 p.m. | 302 | | | | Ate 100 gm. pancreas, 200 gm. meat, 250 cc. milk |
| 6/26 | 9 a.m. | | 1275 | 3.33 | 42.1 | Only 2 pups living, and nursing |
| | 5 p.m. | 386 | | | | Ate 100 gm. pancreas, 200 gm. meat, 250 cc. milk |
| 6/27/35 | 9 a.m. | 280 | 1200 | 2.2 | 26.4 | Placed on insulin |
| | | Less than 0.1 per cent | | | | |

and Hoskins (31) deny this (rabbit). We obtained findings similar to those of Schlossmann in two dogs near term. Most observers interpret the reduction of maternal blood sugar as being due to the passage of insulin

from the fetus to the mother, but Schlossmann and Naeslund interpret their evidence as indicating that the decrease in maternal blood sugar was secondary to hypoglycemia in the fetuses. All evidence at present available on this point is indirect and subject to challenge.

Since we observed greater decreases in the insulin requirement during lactation than during the latter week of pregnancy, we should perhaps not interpret our results as being due to fetal insulin. The effect observed during late pregnancy may be best interpreted as due chiefly to the same effect that operates during lactation. The fetuses in utero as well as

TABLE 6

*Dog 6. Depancreatized 55 hours without showing hyperglycemia. After delivery dog showed hyperglycemia and glycosuria while fasting*

| DATE | HOUR | BLOOD SUGAR | URINE VOLUME | % URINE SUGAR | TOTAL SUGAR | REMARKS |
|---|---|---|---|---|---|---|
| 7/29 | 11 a.m. | 77 | | | | Weight = 10 kgm. |
| | 1 p.m. | Depancreatized | | | | 500 cc. 0.9% NaCl subcut. on table |
| 7/30 | 8 a.m. | 99 | 115 | 3.3 | 3.8 | 500 cc. NaCl subcut. |
| | 3 p.m. | 47 | | | | Ate 300 cc. milk |
| | 10 p.m. | | | | | 40 cc. milk |
| 7/31 | 9 a.m. | 67 | 115 | None | | |
| | 2 p.m. | 73 | | | | |
| | 5 p.m. | 88 | 6 pups born, 2 dead | | | |
| | 8 p.m. | 196 | | | | |
| 8/1 | 9 a.m. | 266 | 400 | Trace | | Removed pups, refused food |
| | 5 p.m. | 260 | | | | |
| 8/2 | 9 a.m. | 232 | 500 | 1.5 | 7.5 | Refused food and water |
| | 3 p.m. | | | | | 500 cc. NaCl subcut. |
| | 5 p.m. | 265 | | | | Refused food |
| 8/3 | 9 a.m. | 230 | 400 | 3.45 | 13.8 | Dog eating sparingly |
| | 5 p.m. | 235 | | | | |
| 8/12 | 1 p.m. | 285 | | | | Dog sacrificed with ether. Autopsy revealed no pancreatic remnants |

nursing pups require sugar and amino acids and their passage either into the fetus or milk will decrease sugar excretion in the diabetic mother.

In this connection it is significant that maternal weight was lost as a consequence of gestation in only one instance (exception: dog 1 lost 1 lb. during the second pregnancy), and that the fetuses used during the latter part of pregnancy from 7 to 12 grams of dextrose or dextrose forming substances daily, as determined by the decrease in glycosuria. If some fetal insulin did pass into the maternal circulation, such is only indicated significantly by the increase in maternal weight in two of the three in-

stances. A complete metabolic study including an analysis of the products of conception would be required to settle this matter.

*Experiment II. Pancreatectomy near term, no insulin given.* It is reasonable to presume that factors may have been operating in the pregnant dogs depancreatized by Carlson late in gestation, which do not operate, or whose operations are suppressed, when even a small dose of insulin is given. For example, the sudden withdrawal of maternal insulin may influence the liver, adrenals and hypophysis, or the placenta may become more permeable to fetal insulin. Since Carlson's work had been questioned, we decided to repeat it.

*Methods.* Six pregnant animals were obtained. When near term, as determined by palpation, the blood sugar was determined and the pancreas removed (ether-morphine anesthesia). The blood sugar and glycosuria was then determined at intervals prior to and after parturition. Food was given as desired by the dog.

*Results.* The results on the six animals are briefly outlined in tables 5 and 6 and the following protocols:

*Protocol, dog 3*

1st day. Blood sugar at 8:15 a.m. was 83 mgm. At 9:00 a.m. *pancreatectomy* was performed. Blood sugar: 1:30 p.m., 181 mgm.; at 12:00 midnight, 258 mgm. (Body weight, 10 kilos.)

2nd day. Blood sugar at 8:10 a.m. was 263 mgm.; urine volume, 250 cc., sugar present. Dog drank some milk. At 7:10 p.m., blood sugar was 263 mgm.

3rd day. Blood sugar at 9:00 a.m. was 262 mgm. Urine volume, 485 cc.; per cent sugar, 2.4; total sugar 11.6 grams. 3:30 p.m., two dead pups born. Blood sugar at 6:00 p.m. was 317 mgm.

4th day. Blood sugar at 9:00 a.m. was 290 mgm. Urine volume, 245 cc.; per cent sugar, 2.7; total sugar 6.6 grams. Dog drank some milk. Blood sugar at 5:00 p.m. was 375 mgm.

5th day. No lactation. Blood sugar: a.m., 286 mgm.; p.m., 272 mgm.; urine volume, 150 cc.; per cent sugar, 6.2; total sugar, 9.3 grams.

6th day. Blood sugar, a.m., 285 mgm.; urine volume, 160 cc.; per cent sugar 8.3; total sugar, 13.3 grams. Dog took 250 cc. milk.

7th day. Blood sugar, a.m., 280 mgm.; urine volume, 320 cc.; per cent sugar 4.1; total sugar 13.1 grams. Dog took 200 cc. milk and 200 grams meat.

8th day. Blood sugar, a.m., 348 mgm.; urine volume, 500 cc.; per cent sugar, 5.0; total sugar 25.0 grams. Dog placed on insulin and used for other work.

*Comment:* Presence of glycosuria due perhaps to too few pups.

*Protocol, dog 4.* See table 5

*Protocol, dog 5*

1st day. Body weight, 10 kilos. Blood sugar, 1 p.m., 75 mgm.; urine volume, 200 cc., no sugar. *Depancreatized at 4 p.m.* Given 500 cc. 0.9 per cent NaCl subcutaneously.

2nd day. Blood sugar, 9 a.m., 90 mgm. Urine volume, 250 cc.; per cent sugar, 0.9; total sugar, 2.2 grams. Dog has distemper and pneumonia. Given homologous antiserum, 4 cc. Blood sugar, 1 p.m., 120 mgm.; at 5 p.m., 130 mgm.

3rd day. Blood sugar, a.m., 125 mgm.; p.m., 105 mgm. Urine volume, 400 cc. containing no sugar. 2nd dose of antiserum given.

4th day. Blood sugar, 9 a.m., 75 mgm. Urine volume 600 cc., containing no sugar. About 10 a.m., 3 *mature pups* were born and the dog ate 150 cc. of milk with some meat. 12 a.m. blood sugar, 75 mgm.; at 6 p.m., 120 mgm. *Three more pups*, 2 living and 1 dead, born at 6 p.m.; 800 cc. N. S. given by hypodermoclysis. Pneumonia present; nasal discharge absent. Animal coughs. Urine volume at 6 p.m., 200 cc., no sugar.

5th day. Blood sugar, 9 a.m., 65 mgm.; 5 p.m., 70 mgm. Animal ate 200 cc. milk and some meat. Urine volume, 75 cc., no sugar. No lactation. Puppies chloroformed.

6th day. Blood sugar, 9 a.m., 50 mgm. Urine volume, 600 cc.; no sugar. Animal ate some milk and meat.

7th day. Blood sugar 9 a.m., 43 mgm. Urine volume, 400 cc.; no sugar. Animal was then given (10 a.m.) 500 cc. of 5 per cent glucose subcutaneously. At 1 p.m. icteric index was 15. Blood sugar 290. Dog vomiting, contaminating the urine specimen.

8th day. Dog chloroformed. Autopsy: Liver very fatty; pneumonia; 0.3 gram pancreas found.

*Comment:* It is difficult to account for the absence of glycosuria after parturition. This was probably due to the fatty liver.

*Protocol, dog 6.* See table 6

*Protocol, dog 7*

1st day. Body weight, 10.2 kgm. Blood sugar at 9 a.m., 124 mgm. *Depancreatized* at 11 a.m. Has a nasal discharge.

2nd day. Blood sugar, 9 a.m., 151 mgm.; urine volume, 170 cc.; no sugar. Gave 500 cc. of N. S. subcutaneously. Nasal discharge.

3rd day. Blood sugar, 9 a.m., 40 mgm.; urine volume, 400 cc.; no sugar. Drank 200 cc. milk, containing 25 cc. corn syrup. *Delivered 6 mature pups at midnight, all living.*

4th day. Blood sugar, 9 a.m., 261 mgm.; vol. urine, 700 cc.; per cent sugar, 8.0; total sugar 56 grams. Drank 500 cc. milk. Nasal discharge. Blood sugar, 5 p.m., 311 mgm.

5th day. Blood sugar, 9 a.m., 312 mgm.; urine volume, 700 cc., 7.7 per cent sugar; total sugar 53.9 grams. Blood sugar, 5 p.m., 278. Refused food. Distemper. Given 750 cc. N. S. subcutaneously.

6th day. Blood sugar, 9 a.m., 280 mgm., urine volume 1000 cc.; 3.8 per cent sugar; total sugar, 38 grams.

7th day. Chloroformed. Autopsy: no pancreatic remnants: distemper.

*Comment:* Depancreatized 60 hours without showing hyperglycemia, which occurred after parturition, however, in spite of distemper.

*Protocol, dog 8*

1st day. Blood sugar, 11 a.m., 72 mgm. *Depancreatized* at 1 p.m.

2nd day. Blood sugar, a.m., 124 mgm. Volume of urine 360; 1 per cent sugar; total sugar 3.6 grams.
Blood sugar p.m., 104 mgm. Given 500 cc. N. S. subcutaneously.

3rd day. Blood sugar, a.m., 155 mgm. Urine volume, 1000 cc.; no sugar. *Three mature living pups born during early a.m.*, 5 more living pups by noon, and 1 more, dead, during p.m. Blood sugar at 4 p.m., 230 mgm.

4th day. Blood sugar, a.m., 271 mgm.; p.m., 280 mgm. Urine volume, 300 cc.; 2.2 per cent sugar; total sugar 6.6 grams. No lactation; pups removed. Dog sacrificed with ether 6 days post-partum. No pancreas found.

*Comment.* Depancreatized 42 hours without hyperglycemia resulting until the birth of 8 pups had occurred.

The results on dog 3 may be considered as negative, although it is significant that only two small dead pups were delivered, the time of in utero death being unknown. The results obtained from dogs 4, 6, 7 and 8 are definitely confirmatory of those of Carlson, Orr and Jones (3). In the case of dog 5 we have no explanation to offer for its failure to develop a hyperglycemia after delivery. In our experience with about one hundred and fifty depancreatized dogs, we (Ivy) have observed only one non-pregnant dog in which hyperglycemia and glycosuria failed to appear after total pancreatectomy. This dog developed distemper the first post-operative day and was treated with homologous antiserum. The distemper, serum and fasting may have been responsible; but in spite of distemper, dog 7 showed glycosuria after delivery. It is to be noted that dog 5 did not lactate (see ref. 7).

*Discussion of results experiment II.* Although our results confirm those of Carlson and his collaborators, such observations do not prove that the failure of a pregnant animal near term to develop hyperglycemia is due to the placental transmission of fetal insulin. Although such an interpretation is the most simple, it is now too presumptuous in view of the recent information pertaining to the rôle played by hypophysis, adrenals and liver in the control of carbohydrate metabolism. Yet, it is certain that the products of conception near term, or the state of pregnancy in some way influences the carbohydrate metabolism, so that pancreatectomy does not result in hyperglycemia in most instances in the dog.

*Experiment III. Pancreatectomy near term, small amount of insulin given.* If the results of Markowitz and Soskin (9), in which they repeated Carlson experiments but gave their two dogs small (3–5 units) doses of insulin, are in general true, then it would appear as if a few units of insulin are sufficient to suppress the influence of the state of pregnancy or the products of conception. If insulin does suppress the influence of the state

of pregnancy, then the improvement we noted in our experiment I is not due to fetal insulin, but due to the passage of sugar and protein into the fetal circulation.

TABLE 7

*Dog 11*

This dog had distemper with edema of the legs when received. It was given homologous antiserum. The distemper and edema cleared after 3 days. Pancreatectomy was performed on the fourth day after the exhibition of serum. Body weight, 14.5 kgm.

Three units insulin twice daily after a meal consisting of 200 grams meat, 100 grams raw pancreas, 100 cc. milk at 8 a.m. and 6 p.m.

| DAY | HOUR | BLOOD SUGAR | URINE VOL. | TOTAL SUGAR OUTPUT | REMARKS | DAY | HOUR | BLOOD SUGAR | URINE VOL. | TOTAL SUGAR OUTPUT | REMARKS |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *mgm.* | *cc.* | *gm.* | | | | *mgm.* | *cc.* | *gm.* | |
| 1st | 7 a.m. | 73 | 620 | | | 15th | 1 a.m. | 118 | 310 | | |
| | 3 p.m. | | | | Pancreatectomy | 16th | 8 a.m. | 74 | 610 | | |
| | 10 p.m. | 105 | | | | 17th | 8 a.m. | 105 | 815 | | *Insulin removed* |
| 2nd | 8 a.m. | 153 | 290 | 2.8 | | | | | | | Diet same |
| | 10 p.m. | 87 | | | | 18th | 8 a.m. | 81 | 280 | | |
| 3rd | 8 a.m. | 220 | 140 | 2.4 | | 19th | 8 a.m. | 225 | 1000 | 14 0 | 7 mature living pups born between 8 a.m. and 2 p.m. |
| | 6 p.m. | 76 | | | | | | | | | |
| 4th | 9 a.m. | 232 | 620 | | Wound not healing well | | | | | | |
| 5th | 8 a.m. | | 260 | Tr.* | Wound not healing well | | 5 p.m. | 320 | | | |
| | | | | | | | 10 p.m. | 444 | | | |
| | 6 p.m. | 400 | | | Wound not healing well | 20th | 8 a.m. | 400 | 730 | 19.7 | |
| | | | | | | | 6 p.m. | 289 | | | Lactation |
| 6th | 8 a.m. | | 880 | 19.4 | Wound not healing well | 21st | 8 a.m. | 400 | 1820 | 112 8 | Permitted mother to nurse 4 pups. Placed dog on 3 *units insulin* twice daily |
| 7th | 8 a.m. | | 500 | 4.0 | | | | | | | |
| 8th | 8 a.m. | | 1100 | 29.7 | | | | | | | |
| | 6 p.m. | 222 | | | | | | | | | |
| | 10 p.m. | 152 | | | | 22nd | 8 a.m. | 266 | 1490 | 123.7 | |
| 9th | 8 a.m. | 230 | 1150 | 4.6 | Incision healing | 23rd | 8 a.m. | 200 | 520 | 9.4 | |
| 10th | 8 a.m. | | 670 | Tr. | | 24th | 8 a.m. | 192 | 800 | 3.2 | |
| 11th | 8 a.m. | | 575 | Tr. | | 25th | 8 a.m. | 210 | 1900 | 5.7 | Dog placed on 8 units insulin twice daily |
| 12th | 8 a.m. | | 520 | 7.3 | | | | | | | |
| 13th | 8 a.m. | 144 | 420 | Tr. | | | | | | | Raised 4 pups |
| | 10 p.m. | 84 | | | | | | | | | |
| 14th | 8 a.m. | 130 | 570 | | Incision about healed | | | | | | |

* Tr. = trace.

*Methods.* Therefore, we repeated Markowitz and Soskin's experiment in which they repeated Carlson's experiments but gave small doses of insulin. Seven pregnant dogs were depancreatized and given 3 units of insulin twice daily. Dog 13 delivered too early to receive insulin, however.

*Results.* The data are briefly summarized in the following protocols and table 7.

*Protocol, dog 9*

1st day. Body weight, 12.5 kgm. Blood sugar at 7 a.m., 95 mgm. Depancreatized at 10 a.m. Blood sugar at 5 p.m., 105 mgm.; at 10 p.m., 113 mgm.

2nd day. Blood sugar at 7 a.m., 82 mgm. Urine output 175 cc. Given 2 units *insulin* at 9 a.m. 12 noon, *hypoglycemic symptoms*; blood sugar, 34 mgm. Given 12 grams dextrose intravenously; condition much improved. 2:15 p.m., labor *1st pup born*. At 4 p.m. dog ate 125 grams meat plus 200 cc. milk. At 8:30 p.m. dog ate 130 grams meat plus 50 cc. milk. Blood sugar just before the meal was 105 mgm. Birth of the pup probably due to hypoglycemic symptoms.

3rd day. Blood sugar 11 a.m., 144 mgm. Urine volume, 210 cc.; no sugar. Blood sugar at 2 p.m., 100 mgm. *Four pups born* between 3 and 6 p.m., one being born dead. 6 p.m., blood sugar, 144 mgm.; 9:30 p.m., 202 mgm. Slight lactation. *Sixth pup* born at 9:30 p.m. Given 3 units *insulin* at 10:30 p.m. with 200 cc. milk.

4th day. Blood sugar 7:30 a.m., 183 mgm. Volume of urine, 520 cc.; 0.2 per cent sugar; total sugar 1.0 gram. Some lactation; 4 pups nursing. Given 3 units *insulin* about 8 a.m. Hypoglycemic symptoms at 9:15, which were controlled with 10 grams dextrose intravenously. Experiment discontinued.

*Comment.* This depancreatized dog was obviously *sensitive to insulin* just prior to parturition and during early lactation.

*Protocol, dog 10*

1st day. Body weight, 16.3 kgm. Blood sugar, 77 mgm. Some milk could be expressed from teats. Pancreatectomy at 8 a.m. 9:30 a.m., blood sugar, 74 mgm.; 3:30 p.m., 90 mgm.; 10 p.m., 121 mgm.

2nd day. Blood sugar, 8:30 a.m., 250 mgm. Only 10 cc. urine present, contained no sugar. Three units *insulin* twice daily. Given after 200 grams meat, 100 grams pancreas, 100 cc. milk. No milk in teats.

3rd day. Blood sugar, 7:30 a.m., 250 mgm. Volume urine, 1780 cc.; trace of sugar. Fed the above meal, giving 3 *units insulin* twice daily.

4th day. No blood sugar estimation made. Volume of urine, 435 cc.; 1.1 per cent sugar; total sugar 4.8 grams. Fed above meal, giving 6 *units insulin* daily.

5th day. Blood sugar, 7:30 a.m., 75 mgm. Urine volume, 760 cc., 0.6 per cent sugar; total sugar, 4.6 grams. Fed meal, giving 6 units *insulin* daily. 4:30 p.m., 1st pup born, living, which mother would not care for, although milk was present in 4 teats. 5 p.m. blood sugar was 222 mgm. 7:30 p.m., blood sugar was 285 mgm. Urine volume, 1340 cc.; 1.5 per cent sugar; total sugar, 20.1 grams. 10:30 p.m., 2nd pup born dead due to impaction of head, which was delivered manually. Lactation. No pups nursing, of course.

6th day. Blood sugar, 7:30 a.m., 360 mgm.; volume of urine 2790; 2.5 per cent sugar, 79.7 grams. Fed and *given insulin* as above.

7th day. Urine volume, 2710 cc.; 1.2 per cent sugar; total sugar, 32.5 grams. Slight lactation.

8th day. Urine volume, 2430; 1.3 per cent sugar; total sugar, 31.6 grams. Milk still present.

9th day. Blood sugar, 8 a.m., 268 mgm.; urine volume, 1050 cc.; 2 per cent sugar; total sugar, 21.0 grams.

10th day. Blood sugar, 8 a.m., 320 mgm.; urine volume, 2670 cc.; 2 per cent sugar; total sugar, 53 grams. Mammary abscess opened.

11th day. Blood sugar, 8 a.m., 219 mgm.; urine volume, 1640 cc.; 2 per cent sugar; total sugar, 32.8 grams.

11th to 19th days. Average blood sugar at 8:00 a.m., 357 mgm.; urine volume, 2263 cc.; total sugar, 54.1 grams. Body weight, 12.3 kgm.

*Comment.* On the same diet and insulin, the glycosuria was much less prior to parturition than after.

*Protocol, dog 11.* See table 7

*Protocol, dog 12*

1st day. Body weight, 11 kgm. Blood sugar, 114 mgm. Pancreatectomy at 5 p.m.

2nd day. Blood sugar, 8 a.m., 145 mgm.; urine volume, 300 cc.; no sugar. Animal did not eat. Given 2 units insulin at 9 a.m. At 1 p.m., *hypoglycemic symptoms*; blood sugar, 25 mgm. Given 25 grams glucose intravenously. At 6 p.m. dog was weak and would not eat. Given 50 grams glucose slowly intravenously. Much improved at 9:30 p.m., blood sugar, 175 mgm.

3rd day. 7.30 a.m., blood sugar, 110 mgm.; urine volume 100; total sugar, 1.3 grams. 12 noon, blood sugar, 165 mgm. Dog expired during delivery. Autopsy: No pancreatic remnants; liver very fatty; 13 mature pups, one being in the lower uterine segment; lungs, red and gray hepatization; cause of death—pneumonia.

*Comment.* This animal was *very sensitive* to insulin due probably to the fatty liver and pneumonia.

*Protocol, dog 13*

1st day. Body weight, 14.5 kgm. Blood sugar, 8 a.m., 80 mgm.; urine volume, 550 cc.; no sugar. Pancreatectomy at 12 noon.

2nd day. 8 a.m., blood sugar, 35 mgm.; urine volume, 400 cc.; no sugar. Delivered 6 mature pups prior to noon. Blood sugar, 2 p.m., 250 mgm.; at 5:30 p.m., blood sugar, 225 mgm. Lactating and all pups nursing. Dog was given 200 cc. milk. At 9 p.m. 200 cc. milk, 125 grams meat.

3rd day. Blood sugar, 300 mgm. Experiment discontinued and animal used for other observations.

*Comment:* This dog delivered too early to use insulin. Blood sugar dropped to 35 mgm. 19 hours after pancreatectomy without insulin, and rose to 250 mm. 4 hours post-partum.

*Protocol, dog 14*

1st day. Body weight, 10 kgm. Blood sugar, 80 mgm. Pancreatectomy at 3 p.m.

2nd day. Blood sugar, 8 a.m., 229 mgm.; urine volume, 240 cc.; total sugar 12 grams. Given *3 units insulin* at 9 a.m. Blood sugar, 12 noon, *54 mgm.*;

at 5 p.m., 95 mgm. Drank 100 cc. milk. Blood sugar at 7:30 p.m., 98 mgm. Delivered 4 pups (small—average 39 gm.) during the night, which soon died.

3rd day. Blood sugar, 8 a.m., 242 mgm.; urine volume, 240 cc.; total sugar, 7.4 grams. Ate food. Experiment discontinued.

*Comment:* A preliminary hyperglycemia, which was converted to a hypoglycemia by 3 units of insulin. Following delivery blood sugar rose to 242 mgm.

*Protocol, dog 15*

1st day. Body weight, 14.5 kgm. Blood sugar 71 mgm. Pancreatectomy at 8:30 a.m. Blood sugar at 12:30 p.m., 111 mgm.; 5 p.m., 108 mgm.; 10 p.m., 86 mgm.

2nd day. 7 a.m., blood sugar, 100 mgm.; no urine. Given 200 grams meat, 100 grams pancreas, and 100 cc. milk with 3 units insulin twice daily.

3rd day. 8 a.m., blood sugar, 156 mgm.; urine volume, 1000 cc.; total sugar, 10 grams. Given 3 units insulin. Dog drank only 100 cc. of milk at 4 and 8 p.m. Second dose of insulin omitted because of listlessness at 10 p.m. Blood sugar was 64 mgm.

4th day. 7 a.m., blood sugar, 60 mgm.; urine volume, 925 cc.; which contained a trace of non-fermentable reducing substances. Three units insulin given with 100 cc. milk, since animal would not take solid food. At 5 p.m. first pup born. Six pups born by 9:30 p.m. Blood sugar at 4 p.m. just prior to labor, 50 mgm.

5th day. 7 a.m., blood sugar, 251 mgm. urine volume, 1520 cc. Given insulin and food, of which dog consumed two-thirds, gave 2nd dose of insulin and meal.

6th day. 7 a.m., blood sugar, 200 mgm.; urine volume, 960 cc., sugar present but not titrated Experiment disontinued. Etherization and autopsy. Viscera normal.

*Comment:* A preliminary slight glycosuria, followed by hypoglycemia when insulin was given. Hyperglycemia followed parturition.

It is to be noted that four of the six dogs (dogs 6, 12, 14 and 15) were sensitive to insulin, although one developed pneumonia post-operatively. Dog 10 is of special interest because it showed hyperglycemia which was not controlled with 6 units of insulin, but only delivered two pups. It, however, showed considerably more glycosuria after parturition than before. Dog 11 was definitely hyperglycemic on 3 units of insulin twice daily, which was due either to the stitch infection or to the early stage of pregnancy at which pancreatectomy was performed, since the hyperglycemia disappeared after the wound was healed and just before parturition.

*Discussion of the results of experiment III.* We do not believe that the foregoing results support the concept that a small amount of insulin suppresses the influence of the products of conception in a dog depancreatized near term. Dog 10 and dog 3 in the second series of experiments had only two pups and were the only dogs, except dog 11, which manifested a significant hyperglycemia prior to parturition, and are analogous to one of

the two dogs observed by Markowitz and Soskin. Their other dog delivered four pups and is more analogous to our dog 11 (table 6).

General discussion. Our results and those in the literature show that pancreatectomy performed in a pregnant dog near term rarely results in hyperglycemia and glycosuria; but, when pregnancy occurs in a depancreatized dog, which is maintained on a uniform diet and insulin, the insulin requirement is reduced only about 20 or 30 per cent. Thus, a substantial difference exists between the metabolism of a pregnant dog depancreatized near term and given little or no insulin, and that of a dog depancreatized prior to pregnancy and maintained on a uniform diet and insulin. The cause of the difference is not clear. We are convinced that it is not due to the trauma or the anesthetic incident to the operation, since the majority of our animals were in good condition and ate after the operation. It would appear that the sudden withdrawal of maternal insulin either results in a change in the permeability of the placenta so that fetal insulin passes into the maternal circulation, or the sudden withdrawal of maternal insulin influences the hypophysis, adrenals or liver in such a way as to prevent hyperglycemia. The observation that the presence of only two fetuses in utero does not prevent hyperglycemia strongly indicates that the agency concerned is quantitative in its action and suggests that the agency is hormonal in nature. The absolute answer to this question, we believe, awaits the discovery of a chemical method for specifically quantitating circulating insulin, or of an active principle in the products of conception other than insulin which prevents hyperglycemia in a diabetic dog.

The dogs in our first experiment which were depancreatized and maintained on a uniform diet and insulin and then became pregnant, more closely simulate the conditions which exist clinically. Taking the similar and adequately studied dog of Markowitz and Soskin, as well as our own, into consideration, it would appear as if pregnancy improves (decreases the tendency to hyperglycemia) the diabetic condition in some dogs and not in others. This is analogous to the clinical observations referred to in the introduction to this paper. The decrease in glycosuria observed in our dogs may be best accounted for by the utilization of dextrose or dextrose-forming substances by the fetuses rather than to the passage of fetal insulin into the maternal blood stream. This view is supported by the fact that the diabetic condition is "improved" to a greater extent during lactation than during the late stage of pregnancy. (Of course, some might say that the anterior lobe may be concerned in the fetal and the "lactation effect.") Yet, this view does not explain those rare patients in which a very marked improvement in the diabetic condition is reported to occur in pregnancy, and the presence of hyperplastic islets in the fetus. Instances of marked improvement, approaching that observed in dogs depancreatized near

term, are more likely to be attributed to the passage of fetal insulin into the mother or to an inhibition of the "diabetogenic" activity of the hypophysis, or to some change in the activity of the adrenals or liver.

## SUMMARY

1. Two female dogs were depancreatized and maintained on a uniform diet and a dose of insulin which permitted a small degree of glycosuria. The effect of three gestation and lactation periods on the amount of glycosuria and the insulin requirement was studied. During the early stage of pregnancy the glycosuria was increased. During late pregnancy the glycosuria and insulin requirement was decreased. A still greater reduction occurred during lactation. The reduction of glycosuria and the insulin requirement late in pregnancy, as in lactation, may be best accounted for by the passage of dextrose and dextrose-forming substances into the fetuses, rather than to the passage of fetal insulin into the maternal circulation. The only indication of an actual improvement in the diabetic condition observed was the gain in maternal weight which occurred in two of the three pregnancies.

2. Pancreatectomy performed near term in pregnant dogs (7 dogs) does not result in hyperglycemia in the presence in utero of three or more mature viable fetuses. This confirms Carlson and his collaborators. Under similar conditions, the administration of a small amount of insulin (3 units twice daily) does not suppress the antihyperglycemic effect which the products of conception near term exhibit (6 dogs); in fact, four out of six dogs were sensitive to insulin. In view of our present knowledge of the rôle that the hypophysis, adrenals and liver play in carbohydrate metabolism, it is now too presumptuous to explain these results as due to fetal insulin. Yet, the products of conception appear to exert their effect in a quantitative manner, which suggests a hormonal agency.

3. The results show that a substantial difference exists between the metabolism of a pregnant dog depancreatized near term and given little or no insulin and that of a dog depancreatized prior to pregnancy and maintained on a uniform diet and insulin. The latter experimental condition is more analogous to that which obtains clinically in the human patient. If our results under the latter experimental conditions are considered jointly with those observed under similar conditions by Markowitz and Soskin, then it would appear that the products of conception near term cause a decrease in hyperglycemia in some but not all dogs.

## REFERENCES

(1) Carlson and Drennan. This Journal **28:** 391, 1911.
(2) Carlson and Ginsburg. This Journal **36:** 217, 1914.
(3) Carlson, Orr and Jones. J. Biol. Chem. **17:** 19, 1914.
(4) Lafon. Compt. rend. Soc. Biol. **75:** 266, 1913.

(5) Simon, Stulz and Aron. Compt. rend. Soc. Biol. **89:** 571, 1923.
(6) Allen. This Journal **54:** 541, 1921.
(7) Markowitz and Simpson Proc. Roy. Soc. Canada **19:** 71, 1925.
(8) Macleod. Carbohydrate metabolism and insulin. London, 1926.
(9) Markowitz and Soskin. This Journal **79:** 553, 1927.
(10) Wiener. Am. J. Obst. and Gynec. **7:** 710, 1924.
(11) Strouse and Daly. M. Clin. North America **9:** 1491, 1926.
(12) Bowen. N. Y. State J. Med. **27:** 351, 1927.
(13) Bowen and Heilbrun. Am. J. Med. Sc. **183:** 803, 1932.
(14) Collens and Shack. N. Y. State J. Med. **32:** 780, 1932.
(15) Lublin. Abst. J. A. M. A. **82:** 1087, 1924.
(16) Fitz and Murphy. Boston M. and S. J. **193:** 1092, 1925.
(17) Parsons, Randall and Wilder. M. Clin. North America **10:** 679, 1926.
(18) Goodman and Camb. Lancet **215:** 862, 1928.
(19) Lawrence. Quart. J. Med. **22:** 191, 1929.
(20) Holzbach. Zentralbl. f. Gynak. **53:** 641, 1929.
(21) McIlroy, Hill and Pillman-Williams. Post-grad. M. J. **6:** 159, 1931.
(22) Joslin. Treatment of diabetes mellitus. Philadelphia, 1928.
(23) Kramer. Am. J. Obst. and Gynec. **30:** 68, 1935.
(24) Pack and Barber. This Journal **92:** 271, 1930.
(25) Gray and Feemster. Arch. Path. and Lab. Med. **1:** 348, 1926.
(26) Allan. This Journal **67:** 275, 1924.
(27) Cuthbert and Ivy. Proc. Soc. Exper. Biol. Med. **32:** 1209, 1935.
(28) Olow. Biochem. Ztschr. **217:** 407, 1930.
(29) Rupp. Arch. f. Gynakol. **143:** 80, 1930.
(30) Schlossmann. Arch. f. exper. Path. u. Pharmacol. **159:** 213, 1931.
(31) Snyder and Hoskins. Anat. Rec. **38:** 28, 1928.
(32) Naeslund. Acta Obstet. et Gyn. Scand. **7:** 25, 1928.
(33) Corey. Physiol. Zool. **5:** 36, 1932.
(34) Gordon. J. Mich. State Med. Soc., March, 1935.
(35) Randall and Rynearson. Proc. Staff Mayo Clinic **10:** 705, 1935.